INVESTIGATION OF TAINT CHARACTERISTICS RELATING TO THE QUALITY AND FLAVOUR OF COFFEE

BY

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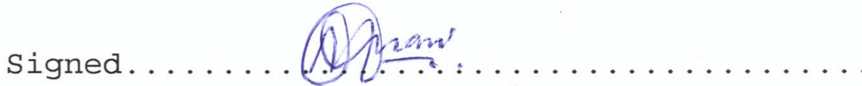

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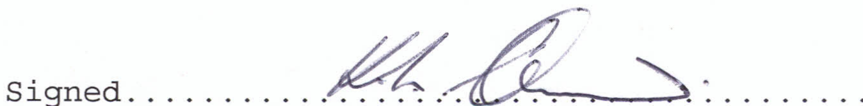

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Kenyatta University.

DEDICATION

To my beloved parents,

Hezron Jonathan Onani and late mother Esther Atieno Onani.

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ABSTRACT

This thesis records the result of an attempt to substantiate an alleged complaint concerning the off-flavour in some batches of coffee as assessed by the trade liquorers of the Coffee Board of Kenya (CBK). The contribution potential of some of the volatile compounds derived from the fungicides used to control some pests and diseases was examined. These volatile substances are possibly produced during the biological breakdown of the fungicide and may be imparted to the coffee causing the taint.

The methods of isolation of the volatile components of the fungicide and later the coffee samples were studied. The coffee samples were ground using a laboratory blender. The ground coffee samples were then extracted using 300ml of dichloromethane in a soxhlet apparatus for a period of eight hours. The excess solvent was evaporated in a rotary film evaporator and the concentrate stored at -20°C in the deep freezer.

The conditions for the analysis of the extracts of the coffee samples were pre-determined. Analysis for the presence of the taint was done using the Sigma 3B gas chromatograph fitted with a fused silica capillary column, BPX5 (0.25), 25m X 0.32mm I.D (SGE). The non-polar separatory phase was 5% phenyl equivalent modified siloxane. The carrier gas, hydrogen was used at a pressure of 6 pounds per square inch (PSI); the instrument was temperature programmed as follows: isothermal at 40°C for 1min, increased at a rate of 5°C/min to 190°C, and held for 5min. A Flame Ionization Detector (FID) was used. All the extracts were analysed using an

on-column injection. Samples (0.025μl) were injected and chromatograms obtained. The retention times (R_t) from the chromatogram were compared to those of the standards run earlier and the results recorded. Analysis of the results supported the circumstantial evidence associated with the possible contribution of the fungicide Prochloraz as responsible for the taint. The taint was observed in all the coffee samples from the areas where the fungicide was used. The fungicide Prochloraz is semi-systemic product and its degradation produces a number of metabolites including 2,4,6-trichlorophenol (TCP). Trichlorophenols are readily transformed to the corresponding trichloroanisole (TCA) *in vivo*. TCP has been shown in its own right to be a fungicide. This active ingredient could be the direct precursor of the TCA found in the tainted coffee samples.

From this study there is a strong evidence that the fungicide Prochloraz produces the observed taint in the coffees studied. Continued usage of this fungicide should be dependant on the basis of further research, a check on application rates and taint appearance.

CHAPTER 1

1.0 Introduction

1.1.0 Coffee - its origin and description

Coffee is one of the members of the family Rubiceae which includes some 400 genera and 500 species, most of which are trees and shrubs. Their occurrence is widespread in the lower region of the tropical rain forest but coffee grows mainly in the highlands. The genus coffea ranges from slender sprawling plants to robust trees with clean trunks and spreading heads growing 10m - 20m high in their natural state but are often pruned to 3m. Pruning is specifically done to maximize yield and facilitate harvesting. Coffea is subdivided into four groups; Eucoffea, Argocoffea, Mascarocoffea, and Paracoffea. Eucoffea contains the most important economical species:

i) Coffea arabica, referred to simply as arabica by the trade and accounts for 76% of the world's commercial coffee with about nine thousand million, mature, cultivated plants.

ii) Coffea canephora, referred to as robusta by the trade and accounts for 23% of the world's coffee with about three and a half thousand million, mature, cultivated plants.

iii) Coffea liberica, accounts for 1% of the world coffee.[1]

Arabica coffee originated from Ethiopia but is now grown in the most important coffee producing countries such as Brazil and

Columbia. Coffea liberica also originated from Ethiopia. Robusta is mainly grown in Africa and Asia having originated from Lomani, an area associated with the Lomani river, a tributary of the Congo river in Zaire[2].

In East Africa, the areas best suited for production of arabica coffee are the slopes of the mountains and highland regions, an area lying between 1400-1900m above sea level. The ideal annual rainfall requirements of coffee range between 1500mm-2250mm which should be distributed throughout the year. A dry period of 2-3 months is however necessary to stimulate flowering. The best soils are well drained fertile volcanic soils. These soils should have a minimum depth of 2m and a pH of 5.3-6.0.

1.1.1 Pests and diseases of coffee

Over 25 pests of arabica coffee have been documented in East Africa. Among the most important pests is the leaf miner, (Leucoptera spp.) whose larvae bore into and feed on palisade tissues of leaves. They form communal mines and these are seen as brown blotches on the upper epidermis of leaves. Other pests include nematodes which attack the roots; aphids, mealy bugs, thrips, capsid bugs, scale insects and antestia bugs which suck berries and terminal buds of branches, causing longitudinal zebra stripping and fan-branching respectively.

The coffee tree is prone to diseases caused by micro-

organisms, the most serious of which are probably coffee berry disease (CBD) caused by *Colletotricum coffeanum* and coffee leaf rust caused by *Hemileia vastatrix* . The symptoms of CBD are observed on flowers and on green and ripe berries. On flowers the first symptoms of CBD is usually a dark brown blotch on the white tissue. The whole flower is invaded in a short time, resulting in its total destruction. On green and ripe berries, small, dark, sunken patches with very small black dots appear and spread rapidly eventually covering the whole of the berry.

For leaf rust yellowish-orange, powdery, round blotches are produced by the fungus on the lower surface of the leaves. In very early stages only pale yellow spots are visible on the leaf but the colour changes in one or two days to yellow-orange as spore production commences. The spores are mainly spread by rain although dispersal by insects also takes place. Germination of the spores occurs in the presence of water and warm temperature conditions. The germinating spores on the lower surface of the leaves penetrate the plant, and timely and careful application of pesticides and fungicides help control these diseases and the pests of coffee.[3]

1.1.2 Harvesting and preparation of coffee for processing

In Kenya coffee is raised from seedlings. A plantation of coffee trees begins bearing 2-4 years after transplanting. The productive life of the trees can be expected to be several years or more depending on variety, climate and husbandry practices. Many of

the coffee trees growing in Kenya at the moment were planted in the early thirties. Sixty years later, the trees are still productive because of pruning, which is a regular practice that encourages new growth from old root stock. The coffee trees have two types of branches, the horizontal branches and the vertical ones. The flowers are produced at the leaf-axils of the horizontal branches. Flowering usually occurs in a flush and is stimulated by the onset of rains following the dry period. Arabica coffee is self-pollinated and from flowering to maturity of the fruit takes about six months depending on local climatic conditions. After the flowers fade, the ovaries slowly develop into oval drupes upto 18mm in length and 10-15mm in diameter. During this swelling, the developing fruit are very prone to attack from diseases. At first the berries are green in colour ripening to bright red (often referred to as cherries) at which stage they are ready for harvesting. Each of the ripe berries contains two beans. The outer skin (exocarp) encloses a tough inner membrane(endocarp). The endocarp, commonly referred to as the parchment encloses the 2 beans. A very thin testa, commonly called silverskin, adheres very closely to each of the beans. Production of coffee can be done by two alternative processes, a 'wet' and a 'dry' process. Where the 'wet' process for production of coffee is practiced, for example in Kenya, harvesting is done by selectively picking the red, ripe berries at intervals of 7-14 days. The berries are delivered to the factory for processing on the same day as they are picked. Where the 'dry' process is used, the coffee cherry is left on the tree

rather longer, and may be stripped from the branches. Leaves and other unwanted material are usually removed in the field and the coffee transported to the drying ground. Most of robusta coffees grown today, and practically the whole of the Brazilian crop, is handled in this way.

1.1.3 Processing of coffee

Preparation of coffee beans for the market entails processing which follows one of two quite distinct methods. The 'natural' or 'dry' process is practiced where the climate is consistently warm and dry following harvest, and where the copious quantities of water required for the 'wet' method may not be available. The 'wet' method is practised where there is plenty of water and sunshine. This method is the most recently developed process of the two and generally leads to higher quality coffee.

The 'wet' process begins with careful harvesting and delivering the cherries to the factory. A preliminary washing is done, during which some inferior quality cherries are separated by floatation. Stones and other foreign bodies are removed at this stage, and the cherries passed to the pulping machine, where they are pulped to remove the exocarp and part of the fleshy mesocarp. On leaving the pulper, the beans pass through a sieve to remove unpulped cherries. The pulp is disposed off as compost or mulch, or may be sun-dried to be used as fuel. The next stage is fermentation, this takes place in large concrete tanks, the

mucilage adhering to the parchment surrounding the bean is broken down by the action of enzymes, and can then be washed away. The beans undergo natural fermentation with the help of enzymes, for example, IAA oxidase and CoA ligase, already present in the mucilage, and aided by those arising from yeasts and bacteria which develop concurrently. The process can take as long as 3 days but can be speeded up by the addition of pectic enzyme preparations. Fermentation is complete when the beans are no longer slimy but have a 'pebbly' feel. They are then thoroughly washed with clean water prior to sun-drying. In most countries, sun-drying is effected on staging consisting of fine-mesh wire netting, others use mechanical driers. During the early stages of sun-drying the coffee has to be frequently stirred or turned, and must be protected from the rain or any dampness by covering it with tarpaulins or matting. The final moisture content expected should not be more than 12%. After this stage the coffee is known as dried parchment. The average annual yield of arabica coffee is 650 kg/ha of dry parchment coffee. Though with good husbandry practices yields as high as 1250 kg/ha of dry parchment can be obtained. In Kenya individual farms have reported yields of upto 3000 kg/ha.[4] The coffee is now ready for the finishing processes which are the final stages of cleaning, hulling and grading.

Hulling involves the removal of the unwanted outer casing or parchment from the bean. The type of machine generally used for hulling, forces the 'parchment' coffee into a confined space by means of a screw feed of reducing pitch where the parchment is

broken by friction and removed by an air current. The coffee grading is discussed in section 1.1.8 of this chapter.

1.1.4 General importance of coffee

Of the worlds agricultural commodities, coffee is of prime economic importance, however, in terms of production, coffee is not one of the worlds' major agricultural products. The export earnings from coffee place it in a unique position which makes it important in international trade. The producer countries are predominantly agricultural and hence coffee is one of the most important sources of their export income. Coffee is mainly sold to the industrialised countries and thus, provides the less developed producing countries with much needed foreign exchange for the development effort.

Coffee is a popular beverage throughout the world partly for its agreeable taste and aroma and partly for its stimulating effect for which caffeine is largely responsible. Levels of caffeine in coffee vary according to species. For example the green robusta coffee contains twice as much caffeine as arabica coffee.

Caffeine has been used therapeutically in the treatment of apnoea, as a bronchial and cardiac stimulant, in the treatment of acne and other skin disorders and migraine headaches. Other established uses involves analgesics, diuretics, weight control aids, allergy relief preparations and alertness compounds.[5]

1.1.5 General economic Importance

Brazil and Colombia are world leading coffee producers. From these two countries coffee provides about 60% and 70% of the total value of exports respectively.[6] In Kenya, coffee provides about 30% of the total value of all the exports.[7]

For international trade purposes, the following three categories of coffee are made:

1. Milds: These are arabica varieties, grown outside Brazil. The important producers are the Central and Latin American countries, Kenya, Tanzania, Ethiopia and Congo.
2. Brazils: These come also from the arabica variety of coffee trees but differ from milds in climatic requirements and processing. On the average milds command higher prices than Brazils.
3. Robusta: These come from the robusta variety and are mainly produced in Africa and Asia. Robustas are used for the manufacture of soluble coffees. On the average robustas yield lower prices than Brazils. In terms of taste and flavours, arabica is softer and milder than robusta.[5]

The Kenya coffee industry mainly produces arabica coffee, which is grown predominantly in the Central and the Eastern Provinces. The Kenya arabica varieties are mild and hence as previously mentioned, command higher prices than those of other countries, except perhaps Colombian coffee. Some robusta are grown

in Nyanza, but this forms a small part of the whole industry. Berries in Kenya are picked during most of the year but the harvest time depends on the climatic conditions of the region and the availability of irrigation procedure. The bulk of the crop is ready for market by November of every year.

1.1.6 Coffee and the Kenyan economy

The Kenyan economy is predominantly agricultural. The majority of people make their livelihood from agriculture. The contribution of agriculture to the national economy has been of immense importance, though Kenyan agriculture exhibits the dual economies often found in developing countries; a subsistence sector mainly producing food crops for personal use and highly developed commercial farms which raise livestock and produce cash crops and food crops.[5]

1.1.6.1 Role of coffee in the economy

Coffee is by far, the most important steady single cash crop and export commodity in Kenya. For a long time, its contribution to the Kenyan export trade has been very high. Tea has been a close competitor, though sisal which used to rank third has since been relegated to fifth position as a result of competition from nylon and other synthetic products. Horticultural and floriculture is a fast developing area and is likely to provide further competition.[6]

Therefore, Kenya's economic development depends heavily on agricultural development which in turn, depends on cash crops such as coffee, so it is necessary for the provision of technical and scientific skill for good husbandry practices so as to ensure high quality production for premium returns. Any negative fluctuation in coffee market price is a major concern to the coffee industry and the national economy.

1.1.6.2 Instability of coffee export earnings

Coffee prices fluctuate widely both in the short term and in the long term trends. In the short term, prices vary from day to day or week to week. Long term fluctuations involve periods ranging from months to months, or year to year and above. The fluctuations mainly involve falling and rising market trends. These are as a result of climatic changes in the field interfering with market supply; pests and disease problems lowering the quality and/or yield; harvesting, processing, storage and transportation, grading and export procedures which can easily bring down the quality or lower the yield.

Fluctuating prices lead to unstable export proceeds and hence instability in the country's income but the extent of the instability depends on how much the country relies on foreign trade. Experiences of cup off-flavour taste is another factor which could lower the coffee premium thereby bringing about instability in the earnings .

1.1.6.3 Coffee quality and flavour

Since coffee is consumed primarily as a beverage, the quality and thereby the flavour in the cup is of primary importance. "Kenyan" mild arabica coffee (produced largely in Kenya) is included in the small group of coffees having the finest quality of all, and therefore has traditionally commanded high market prices in the world coffee markets. To a small extent, it is used by producers of Instant Coffee for blending with the larger quantities of inferior quality robusta coffee.[4] The buyers of the "Kenyan" arabica coffee are therefore largely interested in the quality of the coffee on sale and not the quantity. The survival of the "Kenyan" mild arabica coffee trade as a viable entity thus, depends upon continued production of higher quality coffee.

Very extensive agronomic research programmes in Kenya have considerably increased the yields of coffee per acre, and the effectivity of the production techniques resulting in the production of high quality of the coffee. For several years the African Research Network has engaged its members in active search for quality coffee production. Its Kenyan representative, the Coffee Research Foundation situated in Ruiru, Kenya, has as its principal objective "to promote research into and investigate all problems relating to coffee and such other crops and systems of husbandry as are associated with coffee throughout Kenya, including the productivity, quality, and suitability of land for coffee planting and on matters ancillary thereto.[7]"

This aim ensures that the reputation of Kenyan coffee will remain high in the quality conscious and discriminating world coffee markets. The research has resulted in more efficient processing methods as is highlighted by the fact that properly processed coffee, commands an average market price as high as US$150 per 50kg bag. This is twice the returns obtained from a poorly processed coffee. Because of the premium for the quality, the arabica coffee industry in Kenya pays considerable attention to the grading of its product and use is made of the ultra-violet fluorescence sorting technique[8], to ensure the production of quality grades of coffee.

The sophisticated internal market system adopted requires that all out-turns from the farmers be graded and assessed for quality, and classified by the appropriate Liquoring Department before auctioning. The farmers are then paid on the basis of this classification by the appropriate Central Marketing Authority. Moreover, because of the quota system established by the International Coffee Organisation (ICO), poor grades of coffee are not exported to quota countries. In Kenya, the grading body (CBK) recommends the coffee which the Liquorers estimate to be of an acceptable quality, the least acceptable quality in their score board is graded standard 6. Thus, this coffee is the lowest grade said to be accepted within Kenyan export quota.

It therefore follows that any factor related to coffee quality, no matter how distant it may seem, stands a good chance of justifying some research effort; since factors which affect quality

are bound to have an effect upon the economics of the coffee production.

1.1.7 Volatile components

Flavour can be simply described as being a particular quality or a characteristic that only the tongue can experience. Principally sensations of smell and taste while interacting with other senses comprise flavour. For a substance to evoke taste, it has to come into contact with taste buds, located mainly on the tongue. This presupposes solubility in saliva. For a substance to evoke smell , it has to come into contact with receptors located in the olfactory epithelium in the nasal cavity. This presupposes volatility but this alone is not sufficient, because some volatile substances need to be present in high concentration to be detected while others can be present in quantities as low as of 2 parts in a hundred million million.[3,5] Hence understanding of the odour of a foodstuff necessitates knowledge of the composition of its volatile components both qualitatively and quantitatively. Interference with odour and taste is a direct interference with the volatiles.

Pyrolysis and interactions of components such as sugar, amino acids, organic acids and phenolic compounds result in the formation of the characteristics and flavour of coffee. Most of the volatile components of coffee are derived from non-volatile components of the raw bean which break down during roasting, forming a complex mixture. Several factors dictate the final composition of volatile

components. These include:

1. species or variety
2. climatic and soil conditions during growth
3. coffee processing particularly drying.
4. storage of beans both after harvesting and roasting
5. the time and temperature of roasting
6. roasting equipment.

The volatiles which are present in coffee green beans are found to give rise to its aroma while non-volatiles are responsible for the taste and flavour of the coffee.[5]

Compounds detected in green coffee some of which are precursors of volatiles and most of which increase in concentration during coffee roasting are low molecular weight organic compounds such as: alkenes, ketones, acids and arenes.[9,10,11]; Those whose concentrations increase during roasting due to pyrolysis of non volatile precursors include pyridines, quinolines, pyrroles, arylamines and polyamines; putresane, spermine and spermidine. Aroma volatile components identified to date mainly in roasted coffee number more than 660. These can be broken down into about ten important classes of compounds. These compounds are identified from the coffee, analysed and classified mainly with the help of a low resolution Gas Liquid Chromatography (GLC).

1. Sulphur compounds

These are present at fairly low concentration [12]. Dimethyl sulphide is an essential part of high quality coffee aroma and its presence is said to improve markedly the aroma and the flavour[13] of

coffee. Prolonged brewing yields production of hydrogen sulphide [14,15] which is an off-flavour.

(ii) Pyrazines

At least 81 compounds have been reported [16,17] the abundant ones being methyl and dimethyl derivatives. Alkyl pyrazines are formed during roasting.

(iii) Pyridines

These have a disagreeable and characteristic odour and a sharp taste and therefore their presence is often associated with off-flavours. At least 15 compounds have been detected in roasted coffee. The highest concentration of pyridine has been detected in dark roasted coffee[13].

(iv) Pyrroles

They are structurally similar to pyridines but have agreeable aroma. Therefore they contribute to the quality of coffee[18,19].

(v) Oxazoles

The aromas said to be due to these compounds are described as nutty, sweet and green.

(vi) Furans

There are numerous furans in coffee and they include ketones, aldehydes, esters, alcohols, ethers, acids, thiols and sulphides. When they are present in low concentrations, they posses a sweet smell but high concentrations of the compounds give rise to a burnt smell[18].

(vii) Aldehydes

These are present at relatively high concentrations in freshly

roasted coffee[10].

(viii) Ketones

They vary considerably in aroma character some sweet, some pungent and others fruity.

(ix) Phenols

They are usually present at fairly low concentrations, which increase as roasting continues. They have a smoky, burnt, spicy taste. Sometimes they give rise to clove-like and bitter aromas, which also add an important astringency to the coffee taste[10].

(x) Kahweofuran

This is a heterocyclic sulphur compound which possesses a violent sulphurous odour when pure but on high dilution it develops a pleasant, roasted and smoky note.

1.1.8 Assessment of coffee quality in East Africa.

Coffee quality is largely assessed on the basis of its aroma and flavour by expert coffee tasters and the highest quality beans command a considerable premium. Coffee volatiles are numerous and varied in their aroma quality, potency and concentration, and in the contribution each makes to the overall aroma. At present there is no scientific information which defines coffee quality in chemical terms, and therefore no objective evaluation of coffee liquor or coffee quality can be carried out in Kenya and East Africa as a whole. The present method of assessment employed is based on sensory evaluation to classify coffee for auctioning

purposes, and also as a basis upon which to decide the payout to coffee producers and it is entirely subjective. The sensory evaluation involves assembling a tasting panel. The tasters are presented with the coffee brew samples, at various concentrations and asked to register their finding. The results are compared with the concentrations administered and perception thresholds determined and finally coffee classified.

The assessment of the quality is carried out as follows, a size -(minus) and a shape -(minus) grading of the hulled out-turn is assigned at the curing works, samples are passed on to the Liquoring Department of the Coffee Board of Kenya for quality assessment or evaluation. The liquorer assesses 'raw quality' upon the factors 'size' and 'colour', 'roast quality' upon the factors 'type' and 'centre cut, and 'liquor flavour' (after tasting a cup brew) upon the factors 'acidity' and 'body. Combining these three main subjective classifications, and with consideration of any other remarks of bean defectives and liquor off-flavours and taints, falling under them, the liquorer subsequently arrives at a personal view of the overall quality of the coffee sample. An Out-turn Report Form [from the Coffee Board of Kenya], outlining the main features and illustrating the arbitrary ranked categories of the various quality factors is shown in Fig.1.0.

Figure 1.0: The Outturn Report Form (missing lower parts provides space for class awarded)

COFFEE REPORT AND CLASSIFICATIONS

Agent .... OUTTURN No.

Date .... Ref. No. Mark ....

RAW			ROAST			LIQUOR		
Size	Colour	Quality	Type	Centre-Cut	Quality	Acidity	Body	Flavour
0 Very Bold	0 Bluish	0 Fine	0 Brilliant	0 White	0 Fine	0 Pointed	0 Full	0 Fine
1 Bold	1 Greyish Blue	1 Good to Fine	1 Bright	1 Normal	1 Good	1 Medium	1 Medium	1 Good to Fine
2 Medium Bold	2 Greyish Green	2 Good	2 Ordinary	2 Brownish	2 Fair to Good	2 Light Medium	2 Light	2 Good
3 Medium	3 Greenish	3 Fair to Good	3 Dullish		3 F.A.Q.	3 Light	3 Lacking	3 Fair to Good
4 Mixed	4 Brownish Grey Green	4 F.A.Q.	4 Dull		4 Fair	4 Lacking	4 Harsh	4 F.A.Q.
5 Small	5 Brownish	5 Poor to Fair			5 Poor			5 Fair
6 Very Small	6 Pale	6 Poor			6 Very Poor			6 Poor
	7 Brown	7 Very Poor						7 Very Poor
								8 Foul

REMARKS: (1=Slightly 2=Very)

0 Coated
1 Foxy
2 Light
3 Ragged
4 Underdried
5 Unevenly dried

(1=Few 2=Many)

6 Stinkers
7 Triple centre-cuts
8 OTHER REMARKS:

REMARKS: (1=Slightly 2=Very)

0 Mixed
1 Open
2 Soft

(1=Few 2=Many)

3 Pale
4 Softs

OTHER REMARKS:

REMARKS: (1=Suspicion 2=Slight 3=Bad)

0 Bricky Flavour
1 Chemical Flavour
2 Grassy Flavour
3 Onion Flavour
4 Unclassified Flavour

(1=Slightly 2=Very)

5 Common
6 Earthy
7 Green
8 Musty
9 Sour
10 Strong
11 Unclean
12 Woody

Coffee buyers, for instance from the East African Mild Coffee Trade Association, base their bids upon their own supplementary sample liquorings. The subjective nature of this assessment explains how it is possible for different liquorers to assign different classifications to the same sample of coffee. There could be also wide divergences of opinion, among liquorers, regarding the characteristics of identical coffee samples. Apparently the liquorer's description of liquor characteristics seldom provides any hint of the flavour or tainting agency (see Fig.1.0). Thus whereas the terms 'onion flavour' and 'lacking in acidity' describe recognisable specific physiological sensations or experiences, the terms 'good flavour' and 'foul flavour' give no indication of the exact type of flavour being described. Two coffees can be described as having 'good flavour' and yet the individual flavour characteristics of these two coffees may be utterly different. No known work has been successful in directly linking a taint or desirable flavour to it's chemical precursor. Also, when dealing with coffee which is of a poor overall quality, distinctions are difficult to make, particularly if the coffee samples have undergone similar processing methods. It has been shown[20] how a coffee sample originally awarded class 7 was re-classified as class 2 after careful removal of only 1% of off-flavoured beans.

The need for a more precise and equitable method of assessing coffee quality in East Africa and therefore Kenya, does not require stressing let alone the desirability for an international reference method. Research probing into aspects of objectively determining

coffee quality deserves the effort, particularly where specific chemical compounds have been proposed relating to quality.

1.1.8.1 The importance of off-flavour occurrences in Coffee

From time to time complaints of off-flavours and taint are made about the coffee crops. Though this is infrequent in Kenya coffee, a relatively frequent complaint has been made about Brazilian coffee, which has become known as the Rio taste. This was later discovered to be mainly due to the presence of 2,4,6-trichloroanisole, (TCA see Fig 1.1)

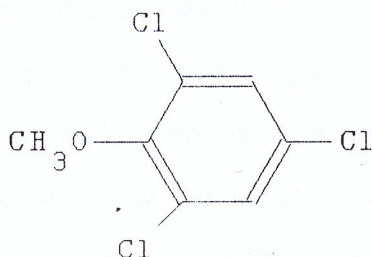


Fig. 1.1 The structure of 2,4,6-trichloroanisole

a very well known off-flavour agent. Off-flavour due to occurrence of TCA has been reported in a number of different foods[21,22,23,24,25]. The origin of TCA may possibly be traced to the usage of chlorophenols as effective wood and wood products, preservatives. Chlorophenols being toxic to microorganisms are used as bactericides and fungicides. They can be used as algicides and herbicides against lower and higher plants and as insecticides and molluscicides against invertebrate and vertebrates. Their toxicity

to man is also worth noting. Other general uses of chlorophenols include; postharvest wash for fruits, slime control for pulp and paper, fumigants for shipping-van interiors, preservative for hardboard and particle board, preservatives for can- end cement, paper and fatty food, sealing gaskets for containers, preservatives in coatings and rubber antioxidant[26].

The chlorophenols present are believed to be susceptable to microbiological methylation and thus may be converted to the corresponding chloroanisoles or they can undergo biological degradation to lower chlorophenols which are methylated afterwards. A number of mould strains have been found to be capable of performing these conversion[23]. An extensive review of TCA occurrence and formation pathways has been reported[27].

The resulting chloroanisoles are important as they have been found to be associated with musty taste in various foods. But the most potent of them all is TCA as it has been identified in quite a number of different foods. Some of the reported cases involve musty flavour in eggs and broilers[21,22,23], chilled meat,[24] packaged rice, flour and fruits,[25] cocoa powder[28], wine[29,30] and brandy[31].

1.1.9 Some coffee fungicides and their usage

Higher coffee production demands proper attention to be given to coffee plants before maturity. This may be achieved by use of chemical control of pests, diseases and weeds. This has led to wide use of several types of pesticides, nematocides, herbicides,

insecticides and fungicides. Use of these important chemicals in the field is never allowed until a chemical to be used is tested for a period of 4-5 years and certified safe. This is carried out by the appropriate Research stations, like Coffee Research Foundation in Kenya established for coffee research.

The majority of commercial fungicides used at present belong to the class known as protectant or surface fungicides. These are usually applied to plant foliage as dusts or sprays. Effective protectant fungicides must satisfy the following conditions:

(a) It must be a fungitoxicant.

(b) It must have very low phytotoxicity, otherwise too much damage will be caused to the host plant during applications.

(c) Generally the fungicide must be able to penetrate the fungal spore and reach the ultimate site of action in the fungus.

(d) It must be capable of conversion into an active fungitoxicant within the fungal spore and must act quickly before the fungal infection penetrates the plant cuticle.

(e) since most agricultural protectant fungicides are applied as foliar sprays, they must be capable of forming tenacious deposits which are resistant to the effects of weathering over long periods.

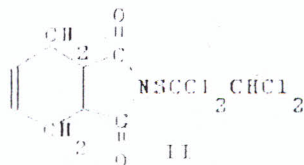
Various fungicides including a range of proprietary 50% (wettable powder (wp)), copper formulations and organic types, many of which are organic based have been largely used.[32,33,36,37] in the coffee industry. These include:

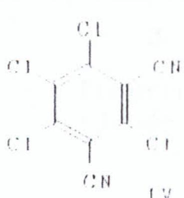
Bordeaux mixture, $CuSO_4.3Cu(OH)_2O$; copper oxychloride, $(CuCl_2.3Cu(OH)_2$; basic copper carbonate, $Cu(OH)_2.CuCO_3$; cuprous

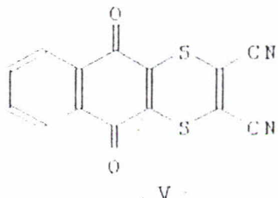
oxide, (Cu_2O); Burgundy mixture, ($CuSO_4.3CuCO_3$) and Cheshunt compound CuSO4 + NH_4OH; Benomyl, (Methyl N-[1-(butyl carbamoyl) 2-benzimidazole] carbamate (I); Captafol, (Ortho-Difolotan), (1,1,2,2-tetrachloro ethylthio) 3a, 4, 7,7a-tetrahydrophthalimide (II); Captan, N(trichloromethylthio) cyclohex-4-ene-1,2-dicarboxyimide (III); Chlorothalonil, 2,4,5,6-tetrachloro-1,3-dicyanobenzene (IV); Dithianon, 2,3-dicyano-1,4-dithia - anthraquinone (V); Fentin hydroxide, triphenyltin hydroxide (VI); Thiabendazole, 2-(4'-thiazolyl)-benzimidazole (VII); Thiophanate, 1,2 - di-(3-ethoxycarbonyl-2-thioureido) benzene (VIII); Thiophanete, Methyl 1,2-di-(3-methoxy carbonyl- 2 -thioureido) benzene (IX); Zineb, Zinc ethylene -1,2- bisthiocarbamate (X); MBC, methylbenzimidazole-2-yl carbamate(XI) and the recently introduced prochloraz[34] (XII) (See structures - Fig.1.2). The fungicides tested and recommended for CBD alone are tabulated in Table 1.1[35].

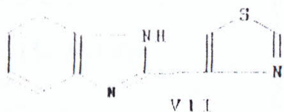
In Kenya copper fungicides are widely used due to their cheapness. They are used by small holder farmers who cannot afford the more expensive organic fungicides. The fungicides are applied as sprays from aqueous suspensions. The spraying usually takes place 10-12 times per annum at rates of 5kg/ha[15].

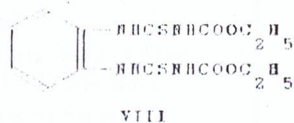
Fig. 1. The structures of some of the chemical compounds used in the coffee industry

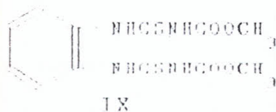


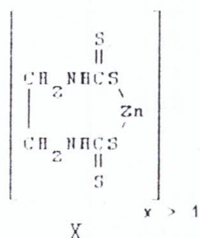


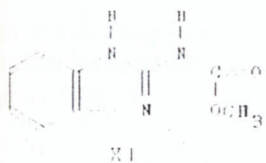












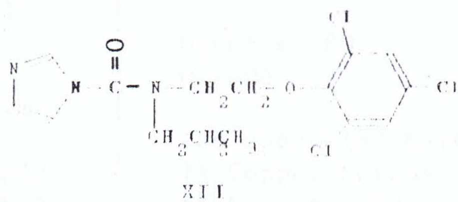


TABLE 1.1

Tested and recommended fungicides for CBD control

Common Names	Proprietary Names (Trade Names)	Formulations	Rate Kg/Ha or l/ha	Interval (weeks) between sprays
Captafol	Orthodifolatan	80% WP	4.4	4
	Moduna	80% WP	4.4	4
	Kenofol	80% WP	4.4	4
	Helmafol	80% WP	4.4	4
	Merpafol	80% WP	4.4	4
	Difolatan 600 flow	600 flow	6.6	4
Chlorothalonil	Daconil 2787-W75	75% WP	4.4	4
	Chlortocaffaro	75% WP	4.4	4
	Griffinil	75% WP	4.4	4
Dithianon	Delan	75% WP	3.3	4
	Delan Sc 750	75% Sc	3.3	4
Anilazine	Dyrene	75% WP	4.4	4
	Dyrene 480 Sc	48% Sc	6.0	4
Anilazine/Copper	Dyrene C47	17/30 WP	9.0	4
Chlorothalonil/ Copper/Maneb	Supanil DG	27/48.3/5.4 Dispersible Granules	7.7	4
Chlorothalonil/ Copper	Dacobre 500	25/30 WP	7.7	4
Prochloraz-Mn	Octave	50% WP	2.2	4
50% Copper formulations	(as specified below)			
(a) Cuprous oxide (RED COPPER)	i) Copper Nordox	50% Cu WP	7.7	4
	ii) Copper Sandoz MZ	50% Cu WP	7.7	4
	iii) Copcel	50% Cu WP	7.7	4
(b) Cupric chloride (Copper oxychloride) (GREEN COPPER)	i) Cobox	50% Cu WP	7.7	4
	ii) Recop	50% Cu WP	7.7	4
	iii) Microcop-50	50% Cu WP	7.7	4
	iv) Funguran	50% Cu WP	7.7	4
	v) Cuprocal	50% Cu WP	7.7	4
	vi) Copsap	50% Cu WP	7.7	4
	vii) Cupravit	50% Cu WP	7.7	4
	viii) Vitigran Conc	50% Cu WP	7.7	4
	ix) Coprado	50% Cu WP	7.7	4
	x) Cuprocaffaro	50% Cu WP	7.7	4
	xi) Vericuivre	50% Cu WP	7.7	4
(c) Cupric hydroxide	i) Kocide 101	50% Cu WP	7.7	4
	ii) Parasol	50% Cu WP	7.7	4
	iii) Champion	50% Cu WP	7.7	4
Copper Sulphate + Lime (Proprietary Pre-mix)	Procida Bordeaux Mixture	25% Cu WP	11.0	4

The current recommendation in Kenya is to spray coffee when it flowers at the beginning of the long rains and then to protect the crop until the weather becomes unfavourable for CBD. This means that five or six applications are necessary during the long rains. Applications should commence again with the onset of the short rains. Only three or four applications are needed in the short rains[16].

Other chlorinated compounds continue to be used and adequate caution must be exercised to avoid problems. For example, 2,4-D (2,4 -dichlorophenoxyacetic acid) must be free from dioxins which may be produced during manufacture. Organochlorine compounds such as DDT are well known for their persistence in the environment. This is due to their resistance to acid, base, heat and oxygen attack. However, DDT has now been banned in most countries of the world. TCA has been reported to cause an off-flavour in various substances. This will be discussed in more detail in chapter 2 section 2.1.0.

The continued use of organochlorine compounds in the coffee industry has led to a suspicion that appreciable quantities of degraded products could interact chemically with any of the volatile components present thereby interfering with aromas and flavour of coffee.

The increased use of various types of fungicides and other agricultural chemicals in the modern world has led to increased possibility of contamination of the crop by those chemicals or their metabolites. In addition the growing environment is also at

risk from accumulation of the chemicals in soil and leaching action into the river systems used for irrigation. In Kenya copper based fungicides are widely used in the control of CBD and coffee leaf rust. Some organic fungicides which are highly effective against CBD have also been recommended one of which is recently introduced organo-carbamate Prochloraz. Degradation of Prochloraz produces a number of metabolites containing the 2,4,6-trichlorophenol moiety (TCP). The next section lists the summary of the two objectives of this study.

1.1.1.0 Objectives of the study

It is the intention of this study to:

(i) investigate the occurrence of taint in some samples of 1991-1992 coffee crop with the aim of providing scientific evidence for the presence of 2,4,6,-trichloroanisole and to examine any possible correlation between the use of Prochloraz and the presence of TCA in those samples.

(ii) convert and upgrade the GLC Perkin-Elmer Sigma 3B gas chromatograph in the Chemistry Department into an instrument capable of accepting capillary columns in order to improve the resolution and sensitivity of the instrument and thus be suitable for detecting the anisole's and related compounds at low concentrations.

In summary, this chapter has been concerned with the origin of

the coffee, preparations, economic importance and quality assessment. The next chapter (chapter 2) will discuss a review of literature concerning TCA and the common methods for the TCA analysis.

CHAPTER 2

2.0 Literature review

2.1.0 A review of 2,4,6-trichloroanisole as an off-flavour agent

For more than thirty years, coffee beans from the Rio area in Brazil have exhibited a special defect affecting green and roasted coffee beans. This defect constitutes a major problem for the quality of the Rio manufactured coffee, which could result in the lowering of the class of coffee and hence its value. The Rio defect has in the past affected upto 20% of the annual crop in Brazil. The off-flavour has been described as medicinal, phenolic or iodine like and has been found to occur occasionally in coffees from other origins[38]. An extensive investigation has been carried out to identify the compounds responsible for the Rio defect and involved isolation of volatiles from green beans by simultaneous distillation-extraction and analysis by capillary column gas chromatograph using detection techniques such as gas chromatograph(GC)-sniffing and gas chromatograph-mass spectrometry (GC-MS). TCA was identified as the most likely key compound for the Rio off-flavour. In some samples of Rio coffee, concentrations have been found to range from 1-100ppb and can be detected by taste at 1-2ppt for flavour by mouth and 8ppt for direct odour perception. Although TCA imparts an off-flavour taste in foods, it has low mammalian toxicit[38].

Several researchers have investigated and reported their recommendations on the role of TCA as an off-flavour agent in different food stuffs. In 1993 a musty off-flavour in several brands of beer were noticed and examination of the beers established that TCA was involved[39]. Lambert et al.[39] reported that lacquered aluminum cans and can ends were an indirect source of TCA in these alcoholic beverages. These workers established that the cans absorbed the volatile TCA during transportation to the filling site from the wood lined shipping containers. The wood linings are preserved using polychlorophenols and these phenols may be biotransformed to yield TCA among other products. The volatile TCA is easily absorbed by the internal lacquer of the aluminium cans and solvent-based sealing compound of the can ends.

By use of GC-MS, TCA was identified in bottled and corked European grape wines as the main component responsible for the musty cork taint occasionally associated with faulty wines[30]. Concentrations of 2,4,6-trichloroanisole found in red and white wines with this distinct off-flavour ranged from 20 to 370 ppt. TCA was not detected in wines of good organoleptic quality, (<2-8 ppt). Organoleptic tests showed TCA to be a very potent compound perceivable in wine at concentrations as low as 10ppt. Buser et al.[25] concluded that TCA and other related chlorinated compounds originated from chlorination of lignin-related substances during the chlorine bleaching used in the processing of cork and that

these compounds are later extracted into the wine.

Whitefield et al.[40] investigated the important off-odour components in tainted jute sacks which were used for the packaging of various cereal grains and flours for storage. They reported that the compounds principally responsible for the musty off-odours in tainted jute sacks, imported from India during 1972-3 and held since then in polyethylene bags at 0°C were TCA and 2,3,4,6-tetrachloroanisole(2,3,4,6-TCA). The origin of the perchloroanisoles was suggested to be either from perchlorophenols and their derivatives used for the rot proofing of jute sacks or perchlorophenols from reaction products of the bleaching of jute fabrics with chlorine. The formation of perchloanisoles in jute sacks was speculated to be probably biological as methylation of chlorophenols is part of the detoxification process of some species of mould which were identified to be present[41]. It was also found that some essential oils contained traces of TCA. It was thought possible that this taint could come also from sacks. The musty taint of the jute sacks was found to be related to the type of mineral oil used in the manufacture of the fibres for the jute sacks. Seifert et al.[42] identified several volatile components in the sacks associated with this mineral oil and the musty taste. TCA was identified to be one of these volatile components.

Other anisoles implicated in taint production are 2,4-dichloroanisole (DCA) and pentachloroanisole (PCA)[43]. However,

these are not so important as their threshold odour detection is considerably higher than for TeCA. In addition the concentration of 2,4-DCA and PCA detected is lower than TeCA and hence these substances are less of a problem.

In 1974, Bemelmans et al.[21] reported a musty taint in chicken eggs and broiler meat. They examined the chicken carcasses and the broiler houses and attributed the taint to perchloroanisoles present in the woodshavings used as litter in the chicken houses. They confirmed the presence of the chlorophenols in relatively high concentration and speculated that, the perchloroanisoles arose through microbiological methylation of chlorophenols in the litter. The other suggestion put forward but which was not investigated was that the contamination was due to the feed.

In 1966, de Groot et al.[44] attributed the presence of 2,3,4,6-tetrachloroanisole and pentachloroanisole in chicken eggs and broiler meat as the likely cause of the observed musty taint, and identified the woodshavings used as litter on the floor of the chicken house as their source.

Gee et al.[45] investigated the metabolism of 2,3,4,6-tetrachlorophenol (2,3,4,6-TCP) by micro-organisms from broiler house litter. They isolated 26 fungal species from broiler house litter and screened their ability to metabolize and methylate 2,3,4,6-tetrachlorophenol. The highest methylation was observed with *penicillium corylophilum* but certain other isolates, notably

that of *P. brevicompactum* metabolized almost all the chlorophenol without forming the corresponding chloroanisole. They concluded that there is more than one route for the metabolism of 2,3,4,6-TeCA, and that the presence of suitable micro-organisms and perchlorophenol could eventually lead to production of perchloroanisole.

Curtis et al.[23] confirmed the cause and origin of musty taint in chicken and broiler systems as 2,3,4,6-TCA formed by microbial methylation of 2,3,4,6-TCP present as wood preservative on shavings used as a litter. Land et al.[22] also reported the cause of musty taint in chickens to be TCP and TCA present in broiler house litter. They demonstrated that pure cultures of microorganisms of fungi isolated from the litter were capable of methylating 2,4,6-trichlorophenol to the chloroanisole whose presence in broiler chickens yielded a musty taint.

TCA was identified by Stoffelsma and de Roos[46] as the unknown trace constituent of a number of essential oils. By comparing gas chromatographic and mass spectral data of the six isomeric trichloroanisoles, they found out that the TCA and the unknown were identical in all respects. In addition, direct gas chromatographic analysis of some oils showed, the presence of a halogen compound having the identical retention time as 2,4,6-trichloroanisole. The origin of the trichloroanisole was of considerable interest, since from general knowledge no halogen compounds had been reported in essential oils and halogen metabolites are rare in higher plants. This added another

dimension on the speculations of the origins of perchloroanisoles. It is possible that soil or plant microorganisms can themselves produce TCP and TCA from metabolites to which they have access. The TCP and TCA could then be absorbed by the plants to contaminate the oils. Alternatively the contamination by TCA and TCP could be due to extraneous sources such as the bags used for the collection and storage of the plant material as was observed in the case of chicken products. Such contamination has also been observed with cocoa powder where chlorophenols and chloroanisoles absorbed from packaging materials resulting in an off-flavour[31]. The source of the chloroanisoles was suggested to be due to a number of moulds isolated from the inner surfaces of the glued seams of the contaminated sacks. Identification of these moulds and subsequent studies showed that some species of *penicillium*, *mucor* and *paecilomyces* were able to methylate 2,4,6-trichlorophenol.

Several complaints about mustiness of rice have been reported[31]. In each case the rice was found to be contaminated with chloroanisoles. Various possibilities for contamination were suggested to occur at some stage between planting of the rice and the consumption of the finished product. It is possible that the soil in which the rice was planted could already be contaminated with cholorophenols and/or chloroanisoles. Alternatively the jute sacks used for transportation of the rice from the exporting country, the holds of ships, warehouses,

wooden pallets, kraft paper bags and cardboard boxes, could have been the source of contamination. Examples of such contamination is well documented[23,31,42,46].

A musty off-flavour in cheese has also been reported[31]. The origin of this taint was speculated to occur during the ripening process of cheese making, during which the cheese is stored on wooden shelves in warehouses. The compounds causing this off-flavour were easily identified as chloroanisoles and were found in rather high concentrations in the plastic outer layer of the cheese which serves as protection for the cheese[31]. Again the rather high concentration of chloroanisoles in the plastic layer was strongly suggested to be as a result of mould development in the wet layer between the shelf and the cheese. These mould can methylate the chlorophenols present in the wood and produce the anisoles which then dissolve in the plastic layer of the cheese.

Whitefield et al.[25] have reported contamination of dried fruit by 2,4,6-trichloroanisole(TCA) and 2,3,4,6-TeCA absorbed from packaging materials. Whitefield and Tindale[47] later carried out an intensive study into some aspects of dried fruit processing and packaging to establish a common link between these cases of mustiness. A common procedure in the dried fruits industry was to store pallets loaded with unformed cartons in the proximity of processing and packaging areas. These areas are frequently cleaned using a combination of high pressure steam and proprietary disinfectants containing high levels of available

chlorine. This combination of a high temperature (>100 °C), moisture, a source of chlorine and a potential source of available phenol in the fibreboard cartons or timber pallets, from the decomposition of lignin, provide good condition for the production of chlorophenols. The study concluded that the possible source of chlorophenols were materials prepared from forest products and use of chlorine-based cleaning agents which could undergo reactions and produce perchloroanisoles.

Spadone and Liardon[48] of the Nestles' Company first investigated the taint of Rio coffee and tried to identify some specific volatile components in the beans with the hope of identifying the components responsible for the characteristic Rio off-flavour. To this effect, they isolated volatile components of Rio and non-Rio green beans using capillary column gas chromatography combined with mass spectrometry and olfactive detection. 2,4,6-Trichloroanisole was identified as a key compound in Rio coffee off-flavour but the origin of the substance in the beans was not known. They suggested as a possible explanation that the TCA could result from the chlorinated phenolic precursor by moulds occurring naturally in Rio beans. It was also speculated that other volatile substances may play a reinforcing role in the production of the Rio off-flavour.

Rio off-flavour was only originally observed in Brazilian

coffees, but recently this defect has been found to affect coffee grown in other countries of the world. In Kenya, in 1992 a problem starkly similar to what has previously been a predominantly Brazilian problem was observed. A survey was conducted by Ojijo et al.[49] during the early part of 1993 to investigate the nature and causes of off-flavours detected in some batches of Kenya coffee. Strange off-flavour were detected mainly in the 1991/92 crop year. They interviewed the majority of the liquorers who described the off-flavour as 'hard or hardish' while some identified another distinct 'taint' from the hard taste. Since its detection, the off-flavour has reportedly featured prominently in several coffee lots delivered at the Nairobi auctions in 1993. The problem was initially observed only in localised stands of coffee on specific farms mainly in the estate sector though some cooperatives too delivered off-flavoured lots. Ojijo et al.[49] suggested that erroneous processing practices, especially at the drying stage, accentuated by relatively wet weather, as possible causes for the off-flavours. Other possibilities were that certain fungicides could impart coffee off-flavours mainly due to their constituents or metabolites. These may be volatile and probably odorous, products of the environmental microflora. Ojijo et al.[49] recommended for further research work to be carried out to furnish unequivocal information regarding the role of fungicides used in the off-flavour defect. It is also worth noting that circumstantial evidence had been put forward suggesting that the fungicide

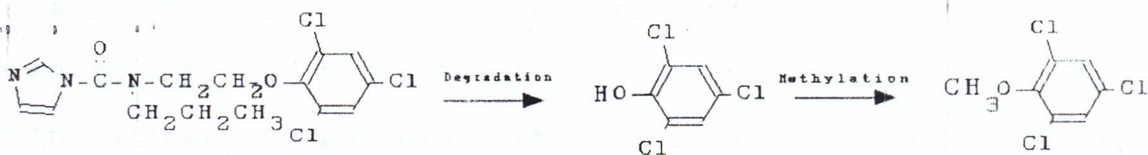
octave may be involved in the production of the taint[4].

Octave is a market name for a complex of manganese dichloride and an organic fungicide Prochloraz, a member of the imidazole class. The fungicide was launched for use in Kenya in 1989 following its testing and recommendation against coffee berry disease contro.[50]. Harris et al.[51] had reported an extensive evaluation programme of the fungicide conducted during 1977-79 period in Western Europe. Tests were reportedly carried out against cereals, lettuce, cotton, french beans, wheat and barley and octave was found an effective fungicide. Knights[52] also reported developments in the use of Prochloraz for tropical fruit disease control and concluded that it was also effective here. Prochloraz has been recommended for use with avocadoes, mangoes, papayas, citrus and bananas. Prochloraz, whose molecular formula $C_{15}H_{16}Cl_{3}N_{3}O_{2}$ has the following structural formula.

Its molecular weight is 376 with a melting point range of 38.5-41.0 °C. It is readily soluble in a wide range of organic solvents but has low water solubility.

Prochlraz (Prochloraz-Mn (50 wp) complex) which is recommended for coffee in Kenya does not possess truly systemic properties, but does show some trans-laminar mobility. It

degrades in the soil to a range of mainly volatile metabolites including the 2,4,6-trichlorophenol moiety. The part of the molecule most probably associated with taint is highlighted (2,4,6- trichlorophenol moiety). Trichlorophenols are readily transformed to the corresponding trichloroanisole (TCA) *in vivo* according to the reaction[27]:



Several chlorophenols, anisoles and higher chlorinated compounds have been reported to produce taint or off-flavours. But TCA is the most important of these compounds since it is the most potent of these taint producing compounds. Because it is the subject of this study, common methods for its analysis will now be reviewed in section 2.2.1.

2.2.0 <u>Common methods for the analysis of 2,4,6-trichloroanisole</u>

Because of the widespread occurrence of TCA, a number of methods of analysis have been developed. The methods mainly used for the analysis of chloroanisoles in different food stuffs involve use of simultaneous distillation-extraction process (SDE), column chromatography (CC), thin layer chromatography (TLC), gas Chromatography (GC) gas Chromatograph-Mass Spectometry

(GC-MS) and high performance liquid chromatography (HPLC). Each of these methods will now be briefly discussed.

2.2.1 Simultaneous distillation-extraction process using the soxhlet apparatus

This is a piece of equipment for the continuous extraction of a solid by a solvent. The material to be extracted is usually placed in a porous paper thimble, and continually condensing solvent allowed to percolate through it and return to the boiling vessel, either continuously or intermittently[53]. Thus concentrating the extract in the boiling solvent.

2.2.2 Micro Kuderna - Danish concentrator

The Kuderna-Danish concentrator (Fig.2.1) was developed for concentrating materials dissolved in volatile solvents, and has wide application in environmental analysis. Typical solvents are methylene chloride and hexane, although many others can be used. The procedure works as follows.

A flask is charged with material to about 40% to 60% of its normal capacity. The column is wetted with about 1ml of solvent before beginning concentration to prevent initial small losses of

material. Boiling chips of some type should be added to the flask and the assembly set over a vigorously boiling water bath. The water level should be maintained just below the lower joint on the flask and the apparatus mounted so that the lower rounded surface of the flask is bathed in steam. When using the volatile solvents, the apparatus is recommended for use in a fumehood. Heavier fractions reflux until the final concentration is collected in the lower tube.

Fig.2.1. Diagram of micro Kuderna-Danish concentrator

2.2.3 Chromatography

Chromatography is a separative process in which a mixture carried in a moving phase (either liquid or gas) is separated as a result of differential distribution of the solutes between the

mobile phase and the stationary liquid or solid, over which the mobile phase is passing[54]. There are three forms of chromatography used for the analysis of 2,4,6-trichloroanisole; These are thin layer chromatography (TLC), column chromatography (CC) and gas liquid chromatography (GLC).

2.2.4 Column chromatography

Impure compounds can be separated by running them through a column of silica gel or other adsorbent using suitable solvents. Zones are formed on the column like rays of light in a spectrum and various components of the mixture are then eluted in a definite order and can then be determined qualitatively and quantitatively.
The fractions from the column can be examined by TLC.

2.2.5 Thin layer chromatography (TLC)

This is a type of adsorption chromatography which is based upon layers of inorganic adsorbent, often silica gel, spread on a glass plate. The recommended thickness for the adsorbent in TLC is 250 μm, and the uniformity is maintained by use of commercial forms of spreaders for the adsorbent slurry. TLC as an analytical technique involves coating the plates with a suitable adsorbent, activating them, spoting of the sample on them, developing in a suitable solvent system, detection with visualising agents,

removal of the separated components if necessary and documentation of the chromatogram.

In 2,4,6-trichloroanisole analysis, the eluates from column and the reference substance (commercial obtained TCA) are spotted on the plate coated with silica gel of TLC grade and then the plate developed using a suitable solvent. Plate development is measured in terms of distance moved by the solvent front and that moved by the compound from the origin. This gives value in terms of retardation factor, R_f, which is defined as the ratio of the distance moved by the compound from the origin to that moved by the solvent front from the origin as shown in the figure below;

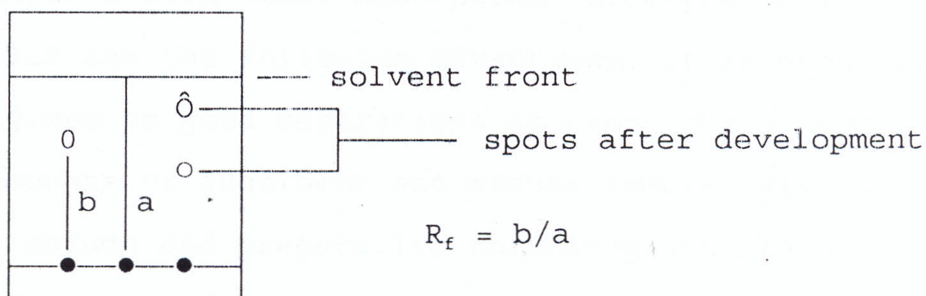


Fig.2.2 TLC Plate Development.

After plate development and detection, if a spot and an authentic material have the same R_f values then the unknown spot is tentatively concluded to be the same as the standard. However, the R_f value of a compound is only approximate and it may lead to ambiguous identification, because the spots on the chromatography are not always sharp, well defined and circular. This makes it hard to achieve reproducibility of R_f values.

Again if a complex mixture is to be analysed by TLC, the major components of the mixture often swamp the colours. Therefore, the R_f values of the minor components are not easily obtained. This makes identification difficult. In such cases the samples may be partially purified by column chromatograph before analysis by TLC.

However, if TLC is the only analytical technique being used, then various solvent systems, stationary phases and spray reagents can be tried out to compare the behaviour of an unknown spot and a given authentic sample. If the two have the same R_f values under the various conditions then the two samples are likely to be identical.

When compared with other adsorption chromatographic techniques, TLC has the following advantages: it is cheaper, quicker and gives as good separations as paper chromatography. Only small amounts of adsorbent and minute samples are used in TLC while in column and preparative chromatography larger quantities of material is needed[55].

2.2.6 Gas Liquid Chromatography (GLC)

In gas chromatography (GC), use is made of a stationary phase which is either a solid (GSC) or a fixed liquid (GLC) packed in a column. There are two types of columns, one is

packed to accommodate larger samples and the second is an open tubular column which accommodates smaller samples but has higher resolution. 2,4,6-trichloroanisole being a highly volatile material, is ideal for analysis by capillary gas liquid chromatography.

The latter type of column does not have any solid support. The stationary liquid phase is coated as a thin film on the surface of the column and is maintained in the column by surface tension. Separation of components is due to their solubility in a mixture; they distribute themselves between the gas phase according to their partition coefficients[56]. The basic components of a GC are shown in Fig.2.3. The working of the GC is as follows.

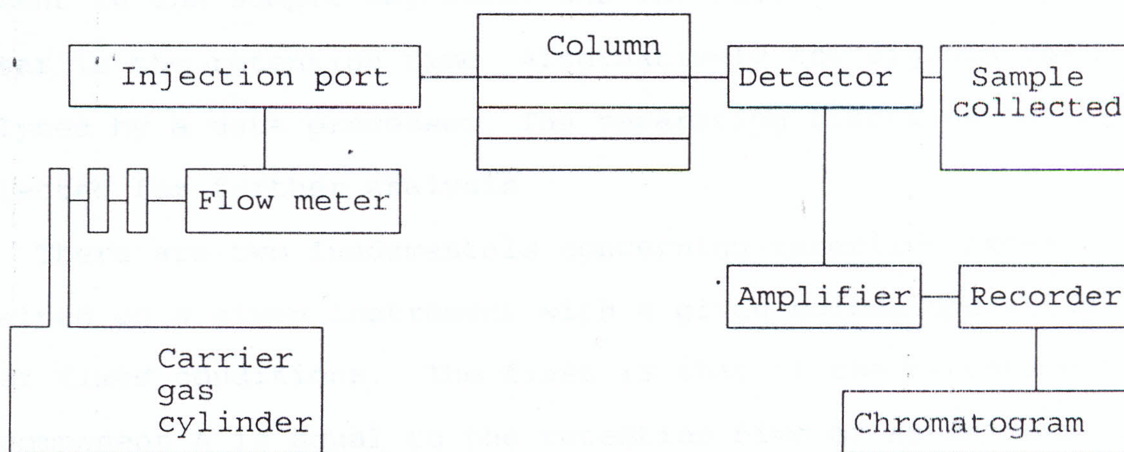


Fig.2.3 Block diagram of the gas chromatograph

An extremely small amount ($\leq 1\mu l$) of a solution of volatile materials is injected into a heated, open tubular column, suitably chosen. The vapours of the injected sample are carried along the tube by an inert carrier gas, usually

argon/methane or nitrogen when an electron capture detector (ECD) detector is used or hydrogen when flame ionization detector (FID) is used. The ECD is a better detector for electron absorbing species and is known to be selective for pesticides and related compounds. FID is the most widely used detector because it is sensitive to most compounds. During this passage, the various components which are present in the original sample are separated into individual fractions which then exit from the opposite end of the column and are monitored in the detector. The electronic signal from this device is fed into a recorder which draws out a set of peaks on moving chart paper and the areas of the peaks correspond to the quantitative percentages of each component present in the sample injected, and the time taken for a peak to appear is the retention time. Alternatively the signals may be analysed by a data processor. The separating fractions may be collected for further analysis.

There are two fundamentals concerning retention times obtained on a given instrument with a given column operating under fixed conditions. The first is that if the retention time of component A is equal to the retention time of an unknown component, this does not prove that the unknown component is A. This prevents gas chromatography from being an exceptional qualitative tool. Secondly, if the retention time of the component A does not equal the retention time of an unknown, then indeed with absolute certainty we can say that the unknown component is not component A[56]. In routine analysis, provided

that the experimental conditions are always the same, the identity of the peaks can be inferred from previous separations, since for a given set of conditions components will always behave in the same way. If the history of a particular sample is known and some knowledge on its composition available, then the technique of peak matching by comparing retention data and peak enhancement may be used. In peak enhancement, an unknown peak can be tentatively identified by adding to the mixture, some of a pure component thought to be identical to the compound responsible for the peak. This is called spiking of the peak in question. If there is an increase in size of the unknown peak, then this is a good indication that the pure substance added is identical. However, this can on occasions lead to erroneous conclusions.

Since there is a possibility of two compounds exhibiting identical retention behaviour, it is more convenient to compare the uncorrected retention time (R_t) for the unknown and the standards run at the same time under identical conditions in multiple columns of different polarity. These values are relatively constant from day to day, laboratory to laboratory, research centre to research centre and can be compared with lists of data computed earlier for standards to make a tentative identification of unknown peaks. However, slight changes of column pressure, temperature, carrier gas flow, the duration a column has been in use and effects of peaks on each other

normally lead to variations in retention data. Wall coated open tubular glass capillary columns (WCOT) are not affected with parameters which influence retention data as long as there is precise temperature and flow control, the indices are highly reproducible[57].

Kovats retention index[57] system for reporting data during GLC analysis is the most precise and repeatable. It describes the retention data of a compound as equivalent to that of a hypothetical n-paraffin hydrocarbon usually containing a mixed number of C- atoms. The index of an unknown compound X is described as:

$$I_x=100\,[n\frac{\log R_x-\log R_z}{\log R_{z+n}-\log R_z}+Z] \qquad (2.0)$$

where

R_x = R_t for the unknown X

R_z = R_t for the normal alkane having Z carbons.

R_{z+n} = R_t for the normal alkane having z+n carbons.

n = the difference in the number of carbon atoms for the normal alkanes.

2.2.7 <u>Capillary column</u>

These are open tubular columns which can be used for smaller samples because they have small diameters ranging between 0.1mm-

1mm and very long length of 25-100m such that better separation is obtained, hence high resolution. There are four types of open tubular columns as at present. These are fused silica (FSOT), Wall-coated(WCOT), Porous layer(PLOT) and support coated(SCOT)[58]. Scientific Glass Engineering **(SGE (UK)** manufactures and supplies a comprehensive range of capillary columns with special requirements and performance advantages. The columns often have wide range of stationary phases, inside diameters, film thicknesses and lengths whose careful choice can help achieve faster and more economical separation. However, it is worth noting that columns with non-polar phases usually have better characteristics in terms of better resistance to oxygen, higher efficiencies and greater maximum temperatures, an example of which is an **SGE** BPX5 column. It has crosslinked phase with a thin film of <1.0μm and a working temperature range of -80-360°C. It is recommended for chlorinated organic compounds. This type of column will be used for this work.

2.2.8 Coupled Gas Liquid Chromatography and mass Spectrometry

When GLC is coupled with Mass Spectrometry (MS), the MS usually acts as a detector to form one of the most powerful analysis techniques available for use. Typical GC/MS has a resolution of ≤ 0.1-1 atomic mass unit (AMU). However, MS is not a very good detector for all compounds. It is destructive and only responds positively to compounds within the limits of their

detectability, even though a complete identification of all compounds is often possible within a short time.

The detection limit of some compounds is often very high and in these cases MS serves as a poor detector. Samples isolated by GLC are introduced into a highly evacuated chamber (pressure of $<10^{-4}$ torr) and bombarded with a beam of elections (70eV), powerful enough to strip away one or more electrons from its molecular orbitals. (see Fig. 2.4) These collisions produce a number of positively charged molecular ions and fragment ions. The ions then pass through a vacuum placed in a magnetic field and an electric field where they are separated according to mass and charge ratio. The influence these ions create on the two fields are amplified and recorded in the recorder.

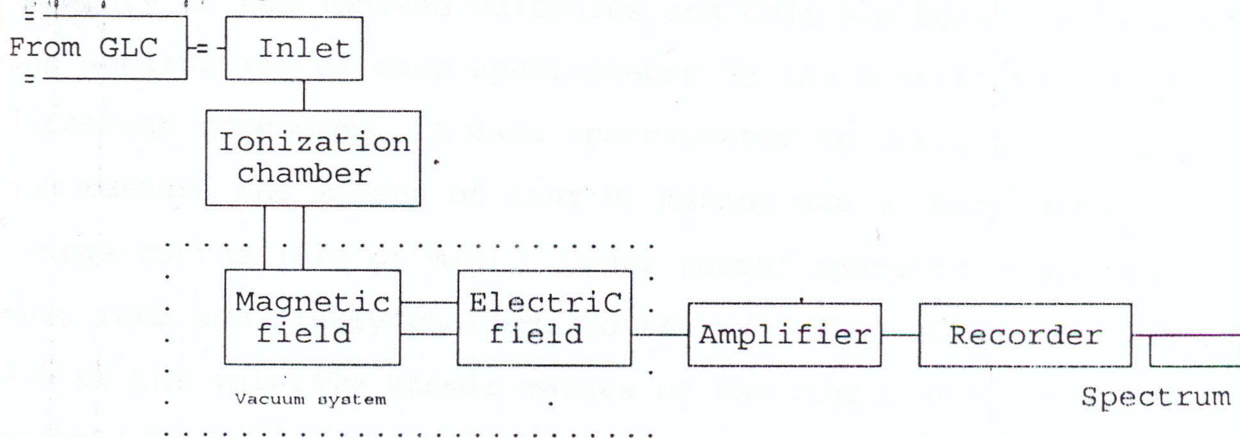
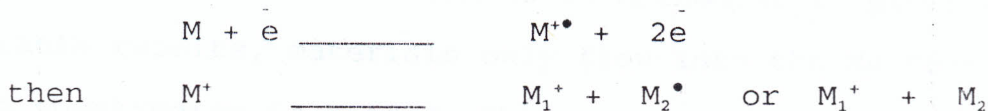


Fig. 2.4 Block diagram of the mass spectrometer.

When a molecule is ionized a molecular ion ($M^{+\bullet}$) is produced;



$$M + e^- \longrightarrow M^{+\bullet} + 2e^-$$

$$\text{then} \quad M^{+} \longrightarrow M_1^{+} + M_2^{\bullet} \quad \text{or} \quad M_1^{+} + M_2$$

The molecular ion (M^+) commonly decomposes to a pair of fragments, which may be either a radical plus an ion, or a small molecule plus a radical cation. Such a series of decompositions when recorded with their mass to charge ratios form a mass spectrum. The molecular ion and any of the fragment ions may decompose by more than one pathway. The extent to which fragmentation takes place along individual pathways is determined by the amount of internal energy originally imparted to the molecular ion and its structure. Hence, the mass spectrum is not simply the fragmentation pattern, but is the appearance of the fragmentation pattern at specified energies and times. The fragmentation pattern found will be characteristic of the identity of the ionized molecules and this has been the basis for the application of mass spectrometry in the identification of different compounds. A mass spectrometer is designed to separate and measure the masses of ions by making use of their mass to charge ratios (m/z or m/e). Under normal operating conditions most ions are singly charged, so the m/e value of an ion is the sum of the relative atomic masses of the components. By establishment of the cracking pattern of a number of pure compounds, the observed pattern permits identification and analysis of unknown complex mixtures[59].

GC-MS is the most recommended method as it gives more reliable results, materials only flow into the MS from GC when a mass spectrogram is needed. This equipment was not available for

this work and therefore can not be used. Instead the Sigma 3B GLC available in the department was used for the analysis. Before using this equipment which was designed to accept a packed column, it was modified to accept a capillary column. A description of how the equipment was transformed will be described in chapter 3 of this thesis.

GLC MACHINE

available in the instrument. The basic instrument from the manufacturer accepts a packed column but the design is such that it can be modified to accept a capillary column, as was done during this work. A description of how the instrument was transformed to accept a capillary column is given in section 3.1.2 of this thesis. The packed column consists of the following:

1) tubing - these are pipes measuring 1-2m with 3.175mm or 6.35mm outside diameter and are made of metals, glass or other inert substance which hold the packing materials.

2) packing material - this is a solid support, usually diatomite or Teflon, which provides the surface onto which the stationary phase is deposited.

3) packing retainers - substances such as glass wool plugs inserted in the tubing ends to keep the packing materials in position.

When material to be separated is injected into the packed column, it is selectively and continuously adsorbed on the packed material. The materials which interact most with the packed material are adsorbed more strongly. The gaseous mobile phase percolates through the stationary phase by capillary action. The components of the injected substance are attracted to and retarded by the stationary phase to varying degrees and as a result, they move along with the mobile phase at varying rates, and are thus separated. It is important to note that use of a

long column yields greater number of theoretical plates and increases column efficiency. The use of stationary phase material that interacts more strongly with the analytes, guarantees better separation power[56].

Over the last fifteen years there has been an increase in use of capillary columns consequently and the use of packed columns continues to decrease. Capillary columns are small diameter open tubes of typical length ranging from 25 to 50 metres. Owing to this, the number of theoretical plates of this type of column is greatly increased and thus, provides for greater resolution. The open unpacked column despite its long length and small diameter, provides for only a small pressure drop over the length of the column. This enables them to have greater capillary activity. Since the stationary phase is applied to the walls of the column in comparison with packed columns, only small amounts of stationary phase can be used. Hence when using capillary columns it is necessary that only small samples are applied.

This work required small sample sizes (μl size) and the results expected were in the range of parts per million or less. Therefore a high resolution chromatography was required than provided by the conventional Sigma 3B gas chromatograph. Because of this, it was therefore, decided to convert the Sigma 3B to an instrument that would take a capillary column. This will be

described in section 3.1.2. Since capillary columns do not accept large sample volumes, a stream splitting system has to be installed, such that excess samples could be vented off. Typically, small samples of about 0.1μL are used with a split ratio of 60:1, in effect reducing the sample size by a factor of about 60. Section 3.1.2 will now describe how the Sigma 3B gas chromatograph was transformed to accept a capillary column.

3.1.2 GLC transformation

In order for the injector to be able to accept a capillary column the existing injector was removed and a new injector installed. The new injector had fittings small enough to connect with the capillary column. Similarly the detector was fitted with couplings of the right size. The equipment used in the conversion of the GLC to accept a capillary column was supplied by Scientific Glass Engineering, SGE Pty Ltd, 1 Potters lane, Kiln Farm, Milton Keynes, MK113LA, United Kingdom. The working of the new unijector body is as follows:

The new unijector body consists of an injector barrel that slides in a brass heat exchanger. The injection barrel is inserted into the brass exchanger until a hexagonal section of the barrel comes in contact with a brass bush. The unijector body is secured in position on the unijector barrel with a set screw. The carrier gas is supplied through a pressure regulator of flow controller to this type of unijector by means of a 1.6mm outside

the split port or septum port by inserting molecular sieve scrubbers in the tubes between the injector and respective valves. The Grob type splitless method requires that the sample be injected into a "closed system"(i.e. a condition when the split ration is not operational). This condition was met by connecting a tee piece from the outlets of the septum purge valve and the split valve using 1.6mm stainless steel tubes. From the tee piece another 1.6mm tube was connected to the dump switching valve, (Fig. 3.2) which ensures the injection is in the closed system.

Once the work on the injection system was completed, the detector end was modified as follows. The ECD which was in the Sigma 3B chromatograph uses radioactive (^{63}Ni) for analyte ionization. Because this detector had not been used for a long period, it was thought necessary to check for radioactive leakage. Using a digital geiger counter (Model XKS-340-010 50-60Hz, 36Va) it was found that the ECD was radioactive leak free.

Following this, the next thing was to prepare the detector head for the capillary column acceptance. This was done as follows: A straight through union was connected to a stainless steel stem through which a 150mm length of 1.6mm outside diameter glass lined tube (GLT) bevelled at one end passes to the base of the union and is locked into the connector with a high temperature graphite ferrule. The internal diameter of the GLT used had to be 0.52mm to fit the vitreous silica column.

Fig. 3.1 Mountings of the split control and Dump switching valve.

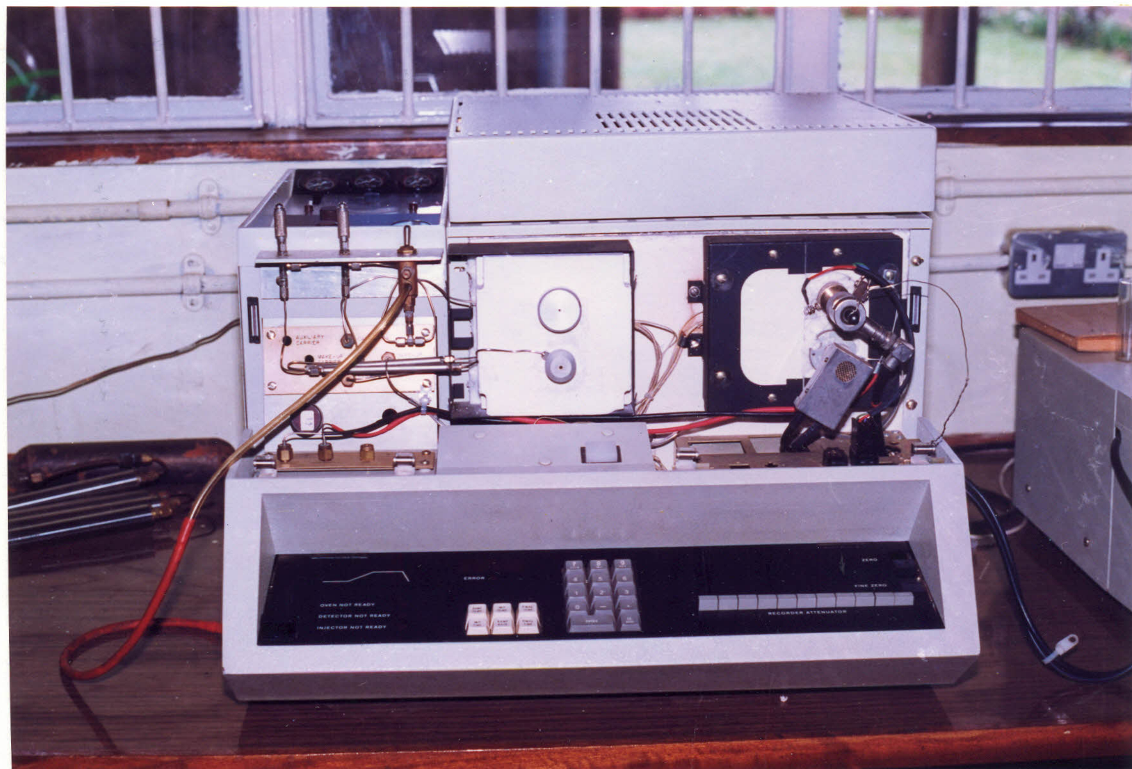


Figure 3.2 The Dump Switching Valve

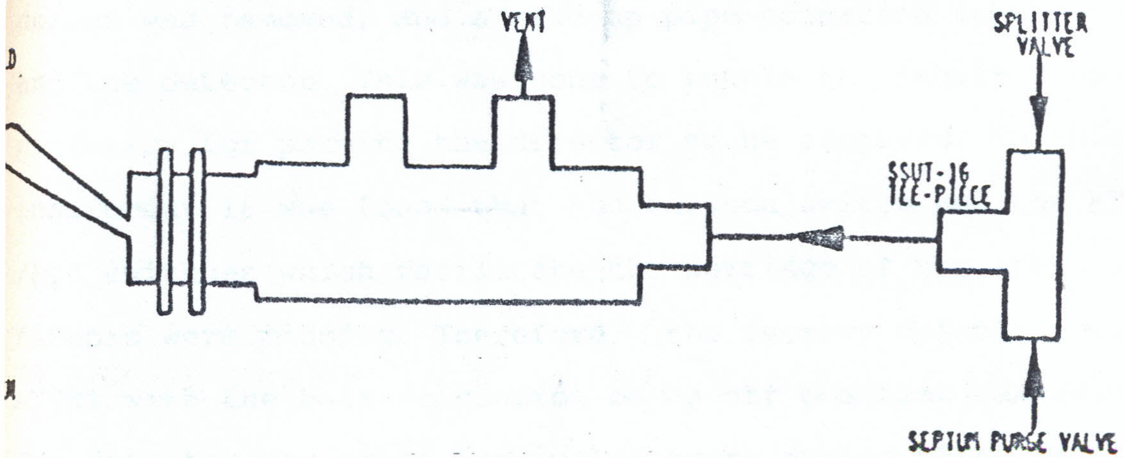
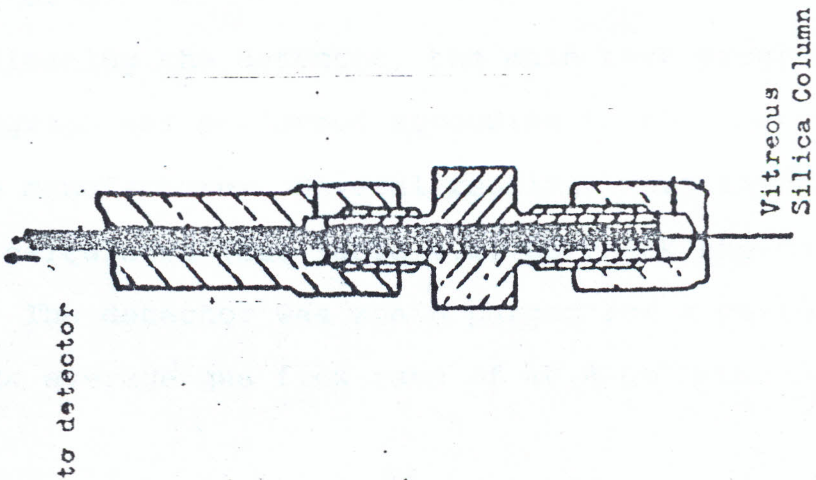


Figure 3.3 The Vitreous Silica Column fitting in the detector

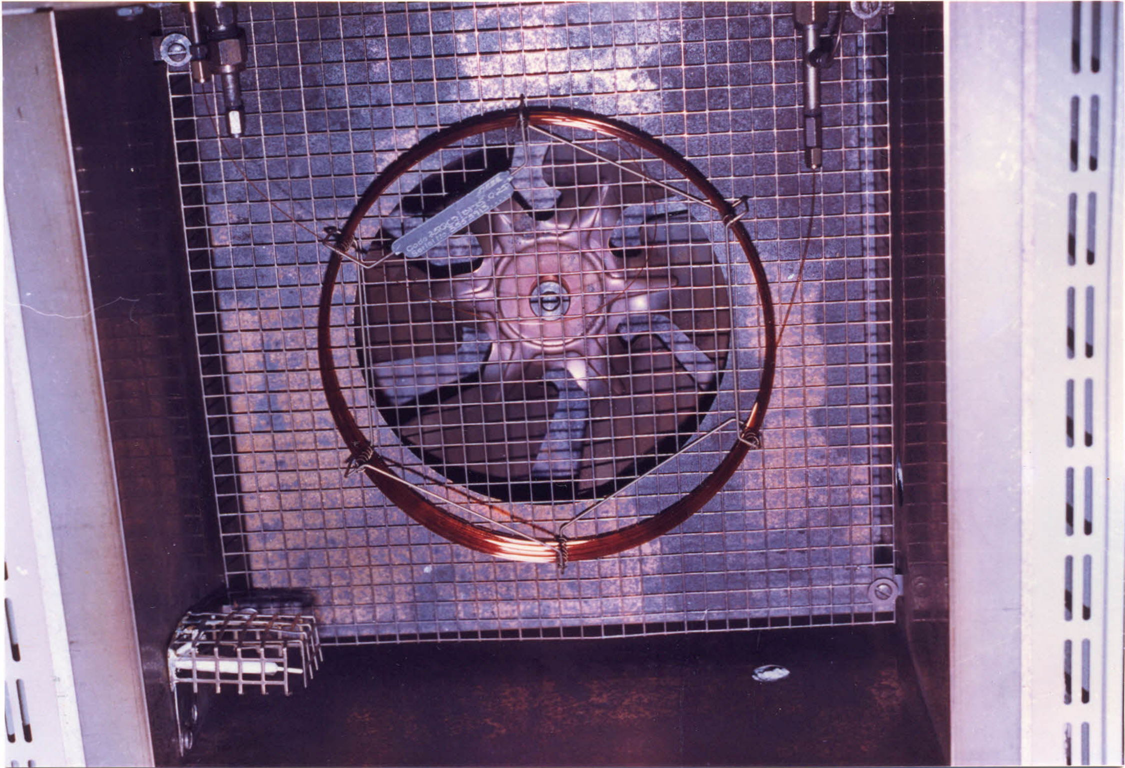


Therefore, the end of GLT inside the union was bevelled to about 85 degrees to ensure an unrestricted gas flow. The 6.35mm stem was locked into the detector fitting using a one-piece ferrule and the stem was held in place using a packed column retainer nut. The vitreous silica column was slid inside the GLT as close as possible to the ECD "jet" as is illustrated in Fig. 3.3.

Following these modifications the next step was to prepare the ECD for decontamination (cleaning). To do this, the capillary column was removed, and a make-up pipe connected to the injector and the detector. This was done to enable the required gas flow necessary for purging the detector to be achieved. In this instrument it was found that the balance switch and the ATT X1 /X10 switches which facilitate the settings of the attenuation factors were missing. Therefore, the factory default setting of ATTX1 with the balance control on an off position was assumed. The detector was baked for twelve hours during which an average gas flow rate of 60 cm^3/min was maintained to clean up the detector. The parameters for baking which were used, were raising the detector temperature to 400 ^{0}C and an oven/injector temperatures of 250 ^{0}C.

After cleaning the detector, the main test program for the gas chromatograph was performed according to the instructions given by the manufacturer. Several readings were taken and pulse frequencies calculated (see Table 4.1) to check the detector performance. The detector was again purged for a period of eight hours with an average gas flow rate of 10.4 cm^3/min. under the

Fig 3.4 capillary fitting



hints manual[60]. Then the manufacturers test sample (TRF C(BP5)) supplied by the column manufacturer was examined under similar conditions to those specified by the manufacturer except for the split ratio which was about 7:1 instead of 60:1 and the detector which was an ECD instead of the FID. A suitable split ratio was investigated by carefully rotating the split control valve to a noted number of turns and the flow rate read from the flow meter. The carrier gas flow rate was measured at the detector vent. The flow meter consisted of two parts graduated in 1ml and 10ml. The snoop bubble passing through the 1ml part was used to measure the split flow rate while the 10ml part was used to measure carrier gas flow rate in ml/s. The flow rate measurements were done twice after an interval of 48 hours. The split ratio was calculated as will be shown in chapter four of this thesis. After establishing the optimum split ratio achievable, the glassware, solvents, chemical standards and the samples to be run on the converted instrument were prepared as will be described in section 3.3 of this thesis.

3.3.0 <u>Glassware and solvents</u>

3.3.1 Glassware

The volumetric glassware used in this work was cleaned by soaking in freshly prepared chromic acid made by dissolving potassium dichromate in concentrated sulphuric acid[63]. It was then washed with a detergent and thoroughly rinsed with distilled

water and left to dry in the oven at 110 °C for 2 hours. When dry and ready for use, the glassware was rinsed with the purified appropriate solvent(see section 3.2.2) before use, to avoid any contaminations.

3.3.2 Preparation of solvents

The solvents ethanol (99%), methanol, chloroform, ether, hexane, dichloromethane and propan-2-one were either bulk or HPLC grade. These were purchased locally. The bulk solvents were double distilled to increase their purity for analytical use. For bulk hexane, an additional treatment was given. It was first refluxed for six hours with acidified potassium permanganate to oxidise the impurities present before distillation. Propan-2-one (500 cm^3) was dried over sodium sulphate, and stored over anhydrous sodium carbonate (250g) overnight to stabilize for use with iodomethane (100ml 96%) in the preparation of further supplies of TCA described in section 3.3.3.1.

3.3.3 Chemical standards

Pure samples of 2,4,6-trichlorophenol (250g, 98%), 2,4,6-trichloroanisole (1g 99%), 2,3,4,6,-tetrachloroanisole (5g 98%) and 2,3,5,6-tetrachloroanisole (5g 98%) were commercially obtained from UK. Stock solutions of 1000ppm were prepared by dissolving 1000mg in a litre of solution. To obtain the standard

solutions, the relevant stock solutions were diluted with appropriate solvents as required. Since the standard TCA purchased was a very small amount. Further supplies were prepared. (see section 3.3.3.1.)

3.3.3.1 Preparation of TCA

The preparation was carried out in the fume cupboard since iodomethane is very volatile and toxic[73]. Anhydrous potassium carbonate (250g) was dried in an oven at a temperature of 140 °C overnight and then cooled in vacuum desiccator. An aliquot of the carbonate (20g) was put in a two necked flask and dry propan-2-one (100ml) and TCP (13.8g) added. Iodomethane (6.9ml) was added slowly via a separating funnel and the mixture refluxed for 2 hours. During the reflux, a drop of the mixture was withdrawn periodically using a glass capillary tube and examined by TLC using silica G and chloroform as an eluant. The R_f observed was compared to that of pure 2,4,6,-TCP dissolved in propan-2-one to check for methylation of the phenol. After complete methylation (from TLC R_f), the mixture was filtered into a beaker and the excess solvent evaporated under reduced pressure. The concentrated filtrate was placed in a deep freezer to crystallize for half an hour. The product was filtered in a pre-weighed Hirsch funnel, washed twice with cold ethanol (25 ml each), and recrystallized from ethanol. The melting point of the prepared TCA and that of equimolar mixtures of the prepared and the

commercial compound were determined and found to be 62-64 °C and 61-63 °C respectively. The implication of this melting point range will be discussed in section 4.2.1 of this thesis. Further confirmation of the product was investigated by comparing a solution of the product and TCP in alcoholic ferric chloride solution (this is a known test for phenols, see also section 4.2.1). Finally the retention time of the product against the commercial compound was checked by GLC. The following section (3.4.0) describes how the minimum amount of the TCA detectable in the GLC and on the TLC was determined.

3.4.0 <u>Detection limit of TCA by GLC</u>.

A detection limit is referred to as the minimum detectable concentration of a substance[56]. Detection limits vary with detectors and instruments. For TCA, the detection limit with both GLC and TLC was examined as follows and the results reported in section 4.2.0.1:

Control samples of purified dichloromethane, hexane and propan-2-one solvents were first run on the GLC. Using pure TCA stock solutions, serial dilutions of upto a factor of 10^5 of the stock solutions was done by pipetting aliquots (10ml) in 100ml volumetric flasks and diluting to the mark using appropriate solvent. Starting with the most dilute solution, samples (0.025μl) were injected in the GC and the chromatogram obtained. From the chromatogram the detection limits were ascertained. The

exercise was repeated using suitable solvent concentrations until the detection limits were accurately determined.

3.4.1 Detection limit by TLC

The TLC technique was used to check for the level of detection of TCA and to determine if it was sufficiently low to confirm the detection established by the GLC.

Starting with the most dilute dichloromethane solution, the solutions were applied to a TLC plate using a capillary tube, and the samples run in a suitable solvent system. (hexane: chloroform 9:1). When developed, the plates were examined under UV light and then sprayed with ceric sulphate solution in sulphuric acid to visualise the separated spots and approximate detection limits established. After estimating the rough detection limits, the exercise was repeated using the suitable solvent concentration (30-40μg) and finally accurate detection limit established.

Section 3.5 will now describe how the extractions were carried out, and the eventual treatments of those extracts.

3.5.0 EXTRACTIONS

3.5.1 Extraction of Prochloraz

Prochloraz (see structural formula in Fig. 2.0) which was obtained from the coffee farmers in Ruiru, Kenya was first extracted. The commercial prochloraz (25g) was put in a soxhlet

thimble and extracted with hexane in a soxhlet apparatus for eight hours. The extract obtained was concentrated in a rotary film evaporator (RFE) and the residual product transferred to a sample bottle. The sample bottle containing the concentrate was then kept in a freezer to prevent any possible chemical reactions that could possibly take place when storage is done at ambient conditions. This extraction was done with the hope of isolating the active ingredient of Prochloraz and any metabolites which may have been responsible for the taint characteristic of the coffee.

3.5.2 Column chromatographic treatment of the extract

A sample of the Prochloraz extract (2ml) was poured into a beaker containing a little column grade silica G. This was thoroughly mixed with aid of a spatula, and the mixture transferred into an already prepared column in a 50ml burette. The preparation of the column in the burette was done as follows: First the burette was plugged with glass wool and then a weighed silica G column grade (120g) was packed in the burette using chloroform. The mass of the silica G used was in compliance with the usual column preparation procedures[64], with a recommended mixing ratio of sample to silica of 1:30. Chloroform was then washed away with hexane. Care was taken to ensure that some few millilitres of solvent was always atop the column to prevent entry of air bubbles which would break the column. In order to

trap any moisture that could possibly interfere with the samples in the column, anhydrous sodium sulphate was poured on top of the column. The sample was then eluted with either of the following solvents (o.s.e):

1. Hexane
2. Hexane:chloroform (1:3)
3. Chloroform with 10% methanol
4. Pure methanol
5. Pure chloroform

The eluates were concentrated using the Danish concentrator and the concentrate spotted on prepared TLC plates.

For the TLC work, preparative TLC plates of 500μm thickness were made by mixing water and Silica G (TLC grade) in a ratio of 1:2, and spreading the slurry on a glass plate with a spreader. This was left to dry and then activated in the oven at a temperature of 110 ^{0}C for a period of 1 hour. The concentrates from the elutions were then spotted on these TLC plates, and developed in a chloroform solvent system in a developing tank. The solvent front was marked after 2 hours and 10 minutes and the plates dried under a vacuum. To visualise the Prochloraz extract concentrate spots, the plates were sprayed with ceric sulphate solution which had been prepared by dissolving 2.5g of ceric sulphate crystals in 65ml of water and adding slowly 35ml of concentrated sulphuric acid. The single spotted extracts were then identified and isolated for crystallization. The isolate obtained was then spotted against a pure TCA to check if they

were related. A run of the samples of the Prochloraz extracts was also performed on the GLC as described in sections 3.6.1.5 and 3.6.2.1.

3.6.1 Studies on the various coffee samples taken from the field

3.6.1.2 Description of the samples

Prochloraz fungicide and twenty six normally processed samples of coffee were obtained from coffee farmers in the Ruiru area of Kenya. These coffee samples contained:

(a) Green* coffee free from taint.

(b) Green coffee containing the taint.

(c) Freshly roasted coffee of (a) and (b).

* Green coffee refers to ripe cherry when pulp and husk has been removed, and the coffee prepared ready for auction.

3.6.1.3 Preparation of coffee samples

The samples under investigation once obtained from the farms were stored in a deep freezer. Before use, 100-150g of these coffee samples were removed and ground using a laboratory blender. For green coffee samples, these were frozen by immersing them in liquid nitrogen to harden and prevent the volatile materials from escaping. The samples were removed from the deep freezer only when required. Roasted coffee beans did not require

the liquid nitrogen treatment as they were already brittle.

A coffee sample labelled 'clean' AC0038 was a coffee sample obtained from the area where the taint was not detected and thus, assumed to be untainted. This 'clean' coffee sample was used to investigate which solvent would be the best for use in the extraction of the coffee samples. Part of the sample was spiked with the commercially purchased pure standards as is described in section 3.6.1.4.

3.6.1.4 Treatment of 'clean' AC0038 coffee sample and the spikes.

A portion of the 'clean' AC0038 (50g) coffee sample was weighed in a round bottomed flask (500ml) and then soaked for one week in 100ml of solutions containing 1000ppm taint promoting compounds made using dichloromethane, propan-2-ol and hexane solvents:

(1) TCA

(2) 2,3,5,6-TeCA

(3) 2,3,4,5-TeCA

(4) A mixture of 10ml each of TCA, 2,3,5,6-TeCA and 2,3,4,5-TeCA.

(5) A mixture of 10ml each of TCA, TCP, 2,3,5,6-TeCA and 2,3,4,5-TeCA.

The solvents were removed using a rotary evaporator, and the samples extracted in appropriate solvents in clean soxhlet

thimbles for four hours and the extracts concentrated under vacuum in a desiccator. The dry samples (20g) were weighed in clean soxhlet thimbles. Then different extractions in different soxhlet apparatus were performed for eight hours with the solvents, propan-2-one, dichloromethane and hexane. The excess solvents from the extracts were again evaporated under reduced pressure. The concentrates were transferred to clean sample bottles with aid of a dropper. These samples were then stored in a deep freezer.

3.6.1.5 Analysis of the spiked samples by GLC

The extracts of the spiked samples (0.5-1.5ml) were removed from the sample bottle and poured in a Danish Concentrator. These were then concentrated to 0.2ml-0.4ml and a GLC run under standardized parameters. The chromatograms obtained were used to investigate the presence of the standards as will be shown in the next chapter. From these runs, it was also possible to identify the solvent which had the best recovery of the spikes.

3.6.2 Extraction of the coffee samples

Each of the coffee samples (20g) were weighed in a clean dry thimble and extracted with dichloromethane (200ml) for eight hours consecutively. Excess solvent was evaporated in a rotary evaporator, and the crude coffee concentrate transferred into a

clean sample bottle. The sample bottle was then stored in a deep freezer for analysis as described in section 3.6.2.1.

3.6.2.1 Analysis of the coffee samples by GLC

The capillary column of the Sigma 3B GLC was thoroughly cleaned by conditioning following the manufacturers recommendations. After this, blank samples of dichloromethane were run on the GLC until no peaks were observed. Then, about 1ml of the crude coffee sample concentrate was concentrated further in the Danish concentrator and the resulting concentrate injected in the GLC. Another crude coffee concentrate (1ml) was cleaned in a 3cm high column packed with column grade silica G (2g) and eluted with chloroform:hexane (1:9) solvent system. The eluent was again concentrated in the Danish concentrator and 1μl samples injected in the GLC. The obtained chromatograms were interpreted for the presence of TCA using the retention times and the results tabulated (Table 4.15).

CHAPTER 4

4.0 Results and discussion

In this chapter, the results and the discussion arising from the work described in chapter 3 are presented. These are presented as follows:

4.1) The results of the GLC transformation and the necessary tests carried out to ascertain the satisfactory functioning of the instrument.

4.2) The results obtained during the search for the best solvent to be used in the extractions in the study. It also discusses the results of the investigations carried out to establish the minimum amount of TCA that could be detected in the chosen solvent, and the results from the extractions and analysis of the fungicide Prochloraz.

4.3) The results of the extraction of the treated and untreated coffee samples

4.5) The discussion of the results and conclusion.

4.10 Sigma 3B GLC and its transformation to accept capillary columns.

The Sigma 3B Gas Liquid Chromatograph was transformed from a basic instrument which only accepts a packed column, to an instrument capable of taking a capillary column. The instrument

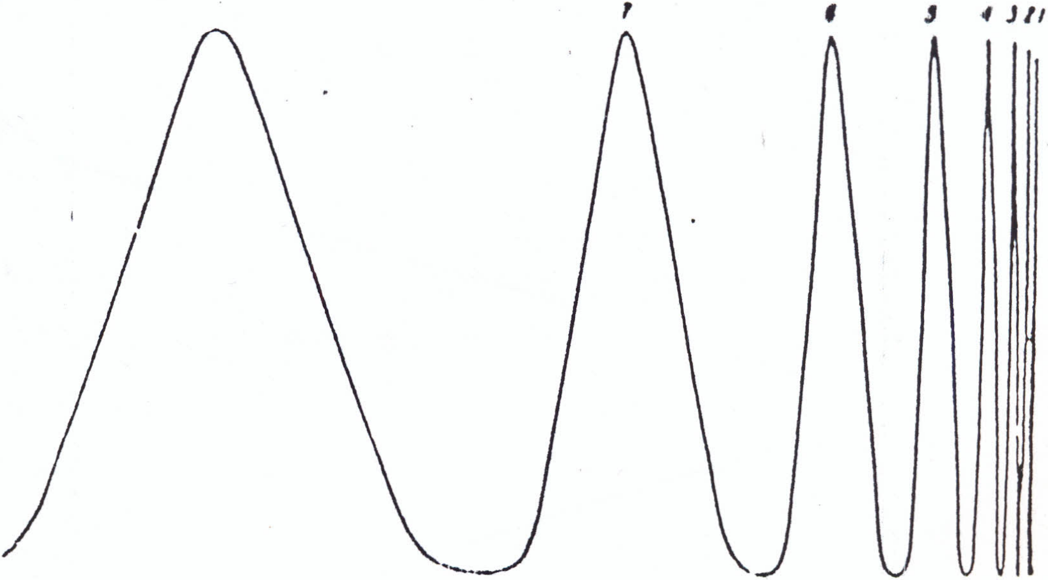
has two detector types, the ECD and the FID. The ECD was initially thought to be a suitable detector for use in this study because of two reasons: first, because of its sensitivity for electron absorbing species such as chlorine containing compounds. Secondly, because it has an extremely high sensitivity (10^{-12} g), which makes it very useful for pesticide analysis, since many pesticides contain chlorine.

After a successful conversion and satisfactory cleaning of the instrument, the main test program was run according to the instructions given in the instrument manual[60]. The peak simulation test produced an 8-peak simulated chromatogram via the detector electronics, similar to the manufacturers report. (compare Figures 4.0 and 4.1 as shown in Table 4.0). The statistical rank correlation coefficient obtained from the two results (1.000 ± 0.002) showed that there are no detectable differences between them. Thus, suggesting that the detector electronics of the instrument were functioning properly and the instrument could be used reliably.

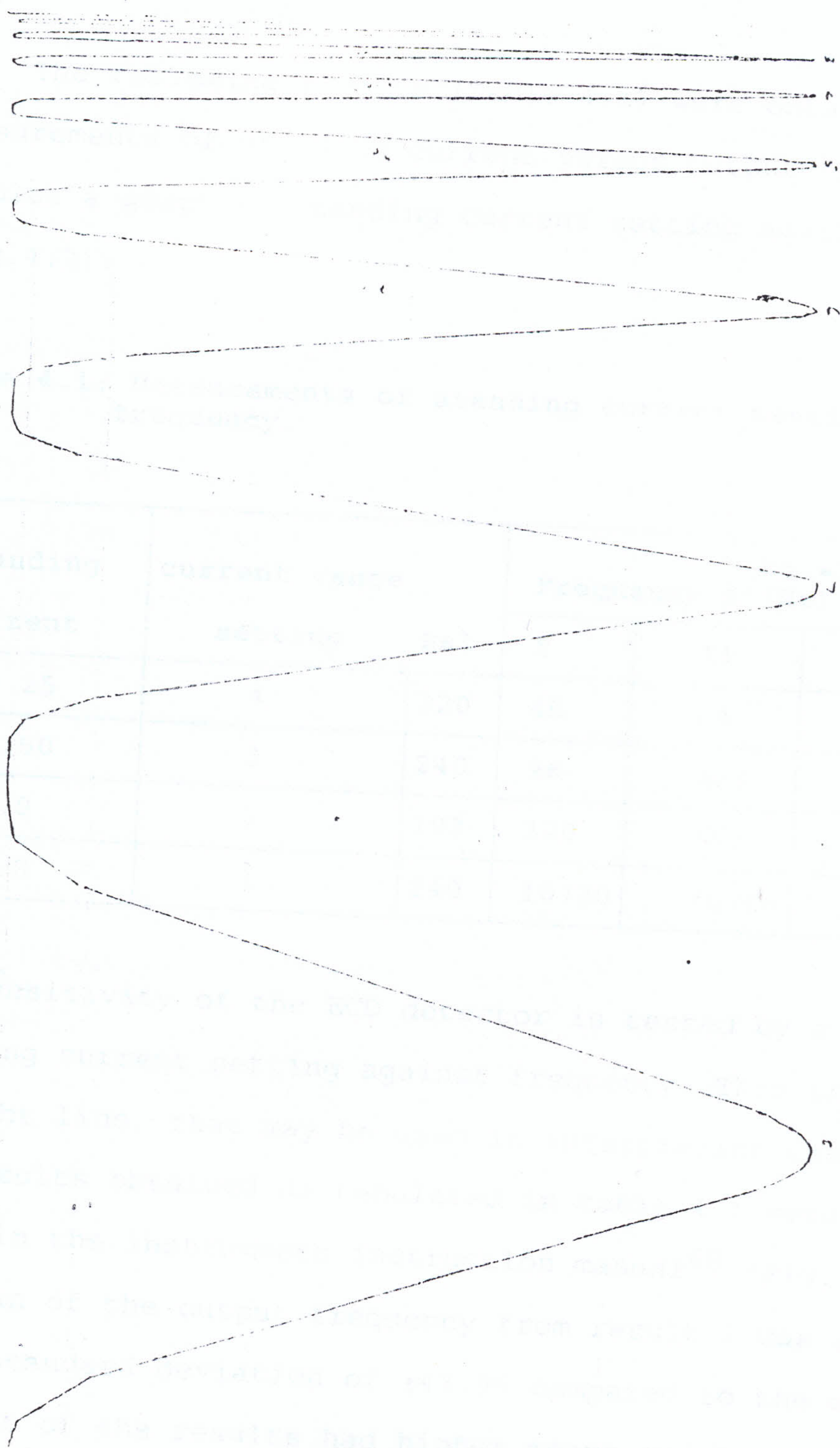
Table 4.0: Results of peak simulation chromatogram.

Peak No.	Width at half-height (Approx)		Mean±S.D
	(I) - Fig.4.0	(II) - Fig.4.1	
1	.7mm	.7mm	0.70±0.00
2	1.2mm	1.0mm	1.10±0.00
3	2.3mm	2.5mm	2.40±0.10
4	4.5mm	4.5mm	4.50±0.00
5	8.8mm	9.0mm	8.90±0.10
6	17.2mm	17.5mm	17.35±0.15
7	34.5mm	35.0mm	34.75±0.25
8	68.5mm	69.5mm	69.00±0.50

Figure 4.0 Sample peak Simulation Recorder Trace



ig. 4.1 Peak simulation recorder trace obtained from running the
ain test program



4.11 The ECD sensitivity test

The following results (Table 4.1) were obtained from the measurements of standing current versus output frequency and used to plot a graph of standing current setting against frequency (Fig.4.2).

Table 4.1: Measurements of standing current setting vs output frequency.

Standing current	current range setting	Bal	bal=240 Frequency f' (Hz) f'=f-bal			
			I	II	III	IV
0.25	4	320	45	64	128	24
0.50	3	240	96	128	160	64
1.0	2	192	320	192	464	64
2.0	1	240	10720	10768	11072	11024

The sensitivity of the ECD detector is tested by a plot of the standing current setting against frequency. This should yield a straight line, that may be used in interpreting the results. When the results obtained as tabulated in table 4.1 were compared to those in the instruments instruction manual[60] (Fig. 4.3). First, the mean of the output frequency from result I was 248 with a lower standard deviation of ±45.96 compared to the other results. The rest of the results had higher means and standard deviations, so a graph of the standing current setting against the output

frequency was plotted using results I. (Fig. 4.2) The graph obtained was found to be negatively skewed, thus indicating that the results obtained from the ECD were not reliable. The reason for the skew can be attributed to the nitrogen gas which was used as a carrier gas. The carrier gas most recommended for this type of work is the argon/methane mixture(95:5), which is expensive and was not available for this work. Therefore there was no alternative to nitrogen gas however, precautions were taken to account for the possible contamination of the nitrogen gas by oxygen. This was done by fixing the gas, disposable purifiers and oxygen indicator in the gas lines.

Oxygen is an ubiquitous impurity, and its presence is particularly important when using an ECD. It is electrochemically active and it interferes with the detector sensitivity. Because it is very electronegative, it can absorb the electrons generated from the detector and produce stable negative ions. Thus, the potentially absorbing species separated during analysis can be denied electrons they need to capture and create the chromatogram signals.

Fig 4.2 Graph of Standing Current against output frequency obtained during the main test program using nitrogen gas

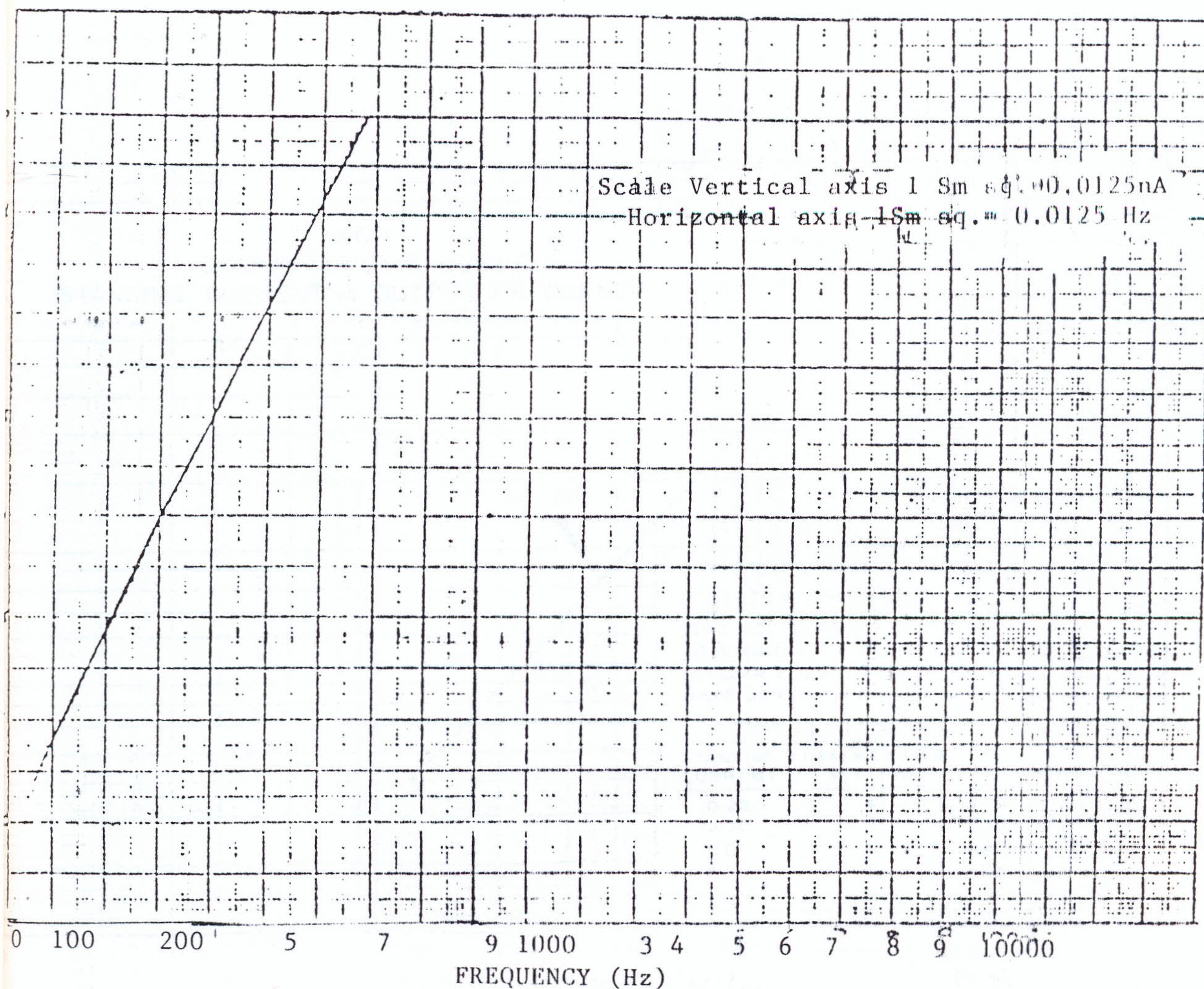
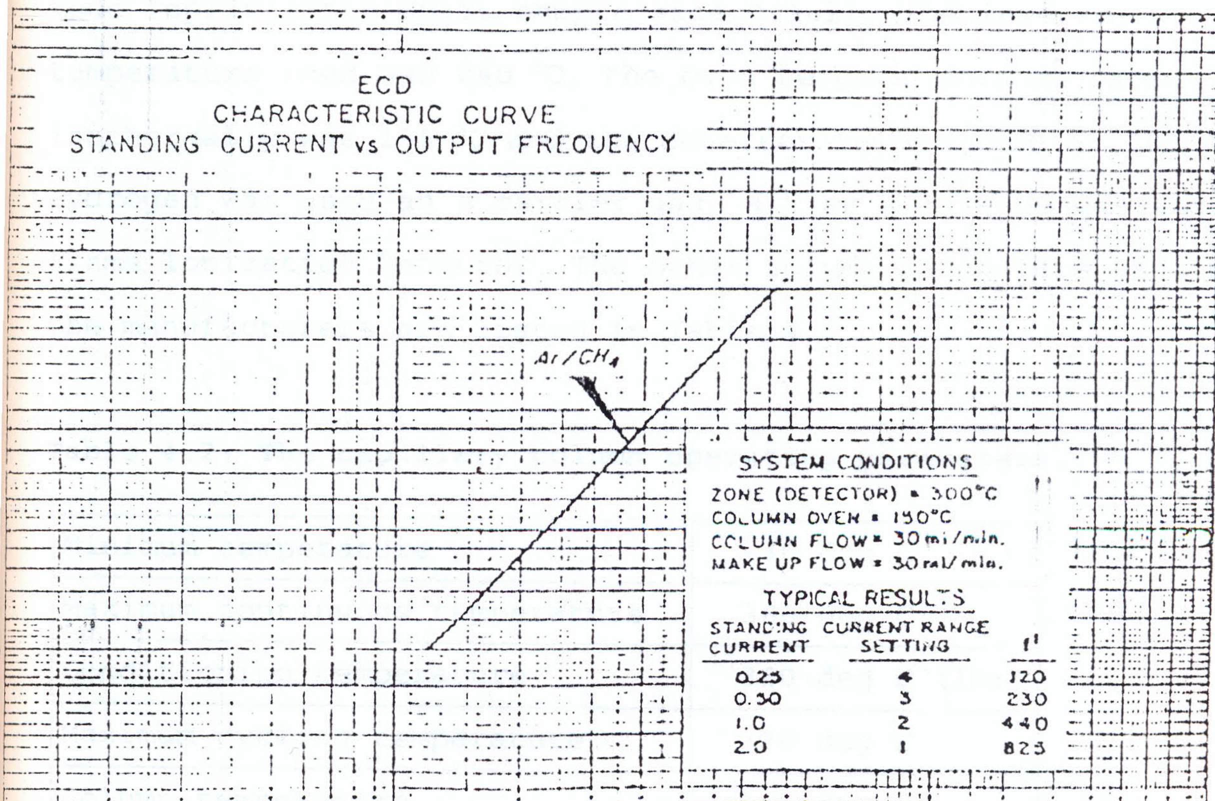


Fig.4.3 Typical standing current against frequency output



4.12 <u>The Sigma 3B capillary column (SGE BPX5)</u>

The Sigma 3B GLC was fitted with a 25m x 0.32mm(i.d.), bonded non-polar phase fused silica capillary column (film thickness 0.25μm). The capillary column manufacturers report sheet indicates that the column performance was tested and the results shown in Fig. 4.5 obtained. The manufacturers used a test sample (TRF C BP5) which they injected in the GLC with a split mode (split ratio 60:1, sample size 0.1μl). The injector temperature used was 240 °C, the oven temperature, operated isothermally was 135 °C and the detector temperature at 380 °C. Hydrogen was used as a carrier gas, air as a make-up gas and a Flame Ionization Detector. The other column parameters used by the manufacturers are listed in Table 4.2.

Table 4.2: The capillary column operating parameters.

Minimum temperature	-40 deg C
Maximum continuous temperature	360 deg C
Conditioning temperature	360 deg C (1hr)
Maximum cycling temperature	370 deg C
Column temperature	135 deg C
Detector temperature	380 deg C
Injector temperature	240 deg C
Inlet pressure was	6.0 PSI.

Because of the high resolving power of the capillary columns, generally the stationary phase specivity is not as critical as in the packed columns. The capillary column had a non-polar stationary phase (BPX5). The non-polar stationary phases tend to have better characteristics than the polar stationary phases in terms of better resistance to oxygen, higher efficiencies and greater maximum temperatures. The BPX5 capillary column (0.32mm i.d.) is regarded by the manufacturers as the best, all purpose, type of column, because it provides good separating power and sample capacity. It is ideal for direct/splitless injection and permits insertion of the selected special type B syringe needle inside the column for on-column injection.

After installation of the column and prior to testing it, the split ratio which is necessary for the satisfactory performance of the column was checked and the results obtained are listed in Tables 4.3 and 4.4.

Table 4.3: Results of the measured flow rates.

No. of turns needle valve	Carrier gas (A) 10ml portion(Sec)	Split gas (B) (1ml portion(Sec)	Split ratio A/B : B/B
1	220.0	0.0	
2	240.0	0.0	
3	240.0	0.0	
4	242.0	55.5	4.4 : 1.0
5	244.0	33.7	7.2 : 1.0
6	245.0	32.7	7.4 : 1.0
7	250.0	33.1	7.5 : 1.0
8	251.0	33.1	7.5 : 1.0
9	250.0	32.2	7.4 : 1.0
10	249.0	33.6	7.4 : 1.0
11	246.0	34.2	7.1 : 1.0
12	246.0	33.9	7.2 : 1.0
13	250.0	34.4	7.1 : 1.0
14	251.0	35.1	7.1 : 1.0
15	250.0	34.8	7.1 : 1.0
16	250.0	34.8	7.1 : 1.0

Table 4.4: Results of the flow rate after 2 Days

No.of turns of needle valve	Carrier gas (A) 10ml portion (sec.)	Split (B) (sec.) 1ml portion	Split ratio A/B : B/B
0	243.0	0.0	-
1	243.0	0.0	-
2	244.0	0.0	-
3	251.0	36.7	6.8 : 1.0
4	249.0	34.9	7.1 : 1.0
5	252.0	35.0	7.2 : 1.0
6	252.0	36.8	6.8 : 1.0
7	252.0	36.8	6.8 : 1.0
8	250.0	33.1	7.5 : 1.0
9	251.0	33.1	7.5 : 1.0
10	250.0	32.2	7.4 : 1.0
11	249.0	33.6	7.4 : 1.0
12	246.0	34.2	7.1 : 1.0
13	246.0	34.3	7.1 : 1.0
14	250.0	34.4	7.1 : 1.0
15	251.0	35.0	7.1 : 1.0

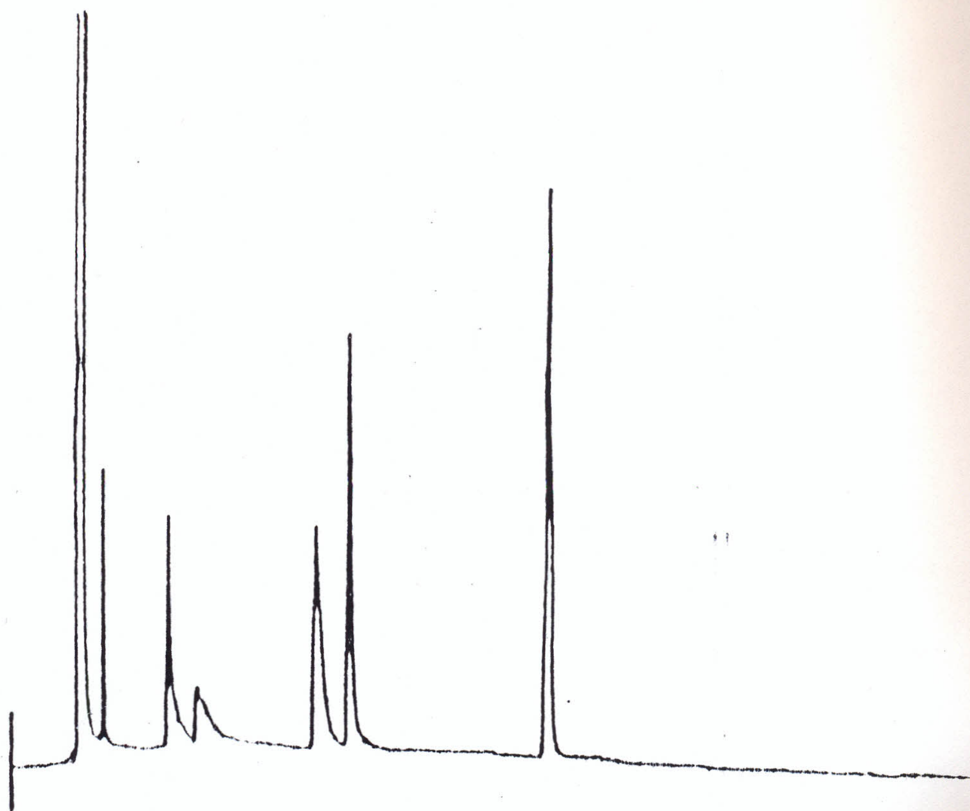
The split ratio is obtained by dividing the volume of the gas coming out of the split port (regulated by split valve) by the volume of the carrier gas being emitted from the detector vent. The results yielded a split ratio of 7:1. This ratio is not comparable to that used by the column manufacturers (60:1)(Table 4.2) probably due to the difference in the type of the instrument used. On the other hand the recommended standard split flow which could neither be lowered nor increased was 0.5 cm^3/min. This implied that once the split flow is set, then it is only the carrier flow rate which was left open for regulation in order to achieve the required optimum split ratio. The split ratio used (7:1) was very low, which would allow a lot of sample to flow through the column and could result in the column overload. If the column is overloaded with the sample, its separation power becomes lowered and tailed chromatograms (chromatograms with external zone broadening) will eventually be obtained. However, this problem was curbed by injecting small samples of $\leq 0.025\ \mu l$, this is an injection which is four times lower than the recommended injection volumes.

The performance of the capillary column was tested using the test sample (TRF C BPC) under similar conditions to those specified in the manufacturer's manual except that the Electron Capture Detector was used. Poor results (see Fig. 4.6) were obtained. An attempt was made to improve the functioning of the instrument, by splitting the inlet and the make-up carrier tubes

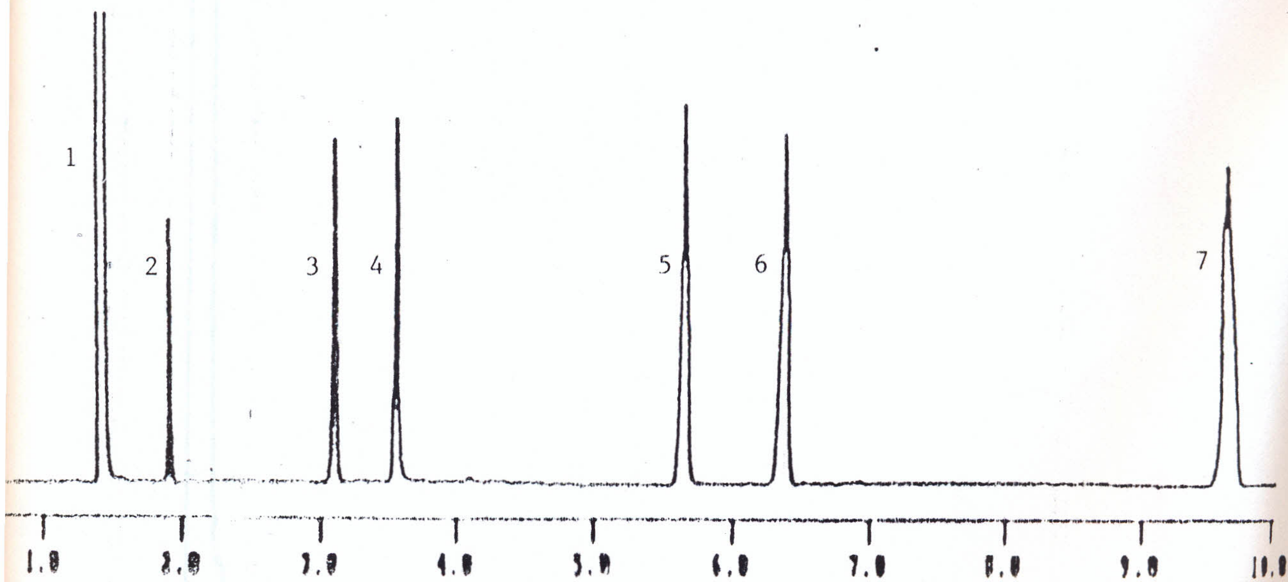
to have them operate independently. Hydrogen gas was used as the carrier and the nitrogen gas as the make-up gas. This gave only a very slight improvement on the results of (see Fig. 4.7), and at this stage it was decided to use the FID in place of ECD. The advantage with the FID is that it allows for the use of chlorinated solvents in the extraction process and does not respond to small volumes of water. The make-up gas was then changed to medical air necessary to facilitate the hydrogen combustion.

After several instrument tests, the conditions shown in Table 4.5 were found to give results (Fig. 4.4), which were comparable to those obtained by the column manufacturers (Fig. 4.5). These were therefore adopted for this work.

4.4 Result obtained after several column performance tests using the test sample TRF BP5

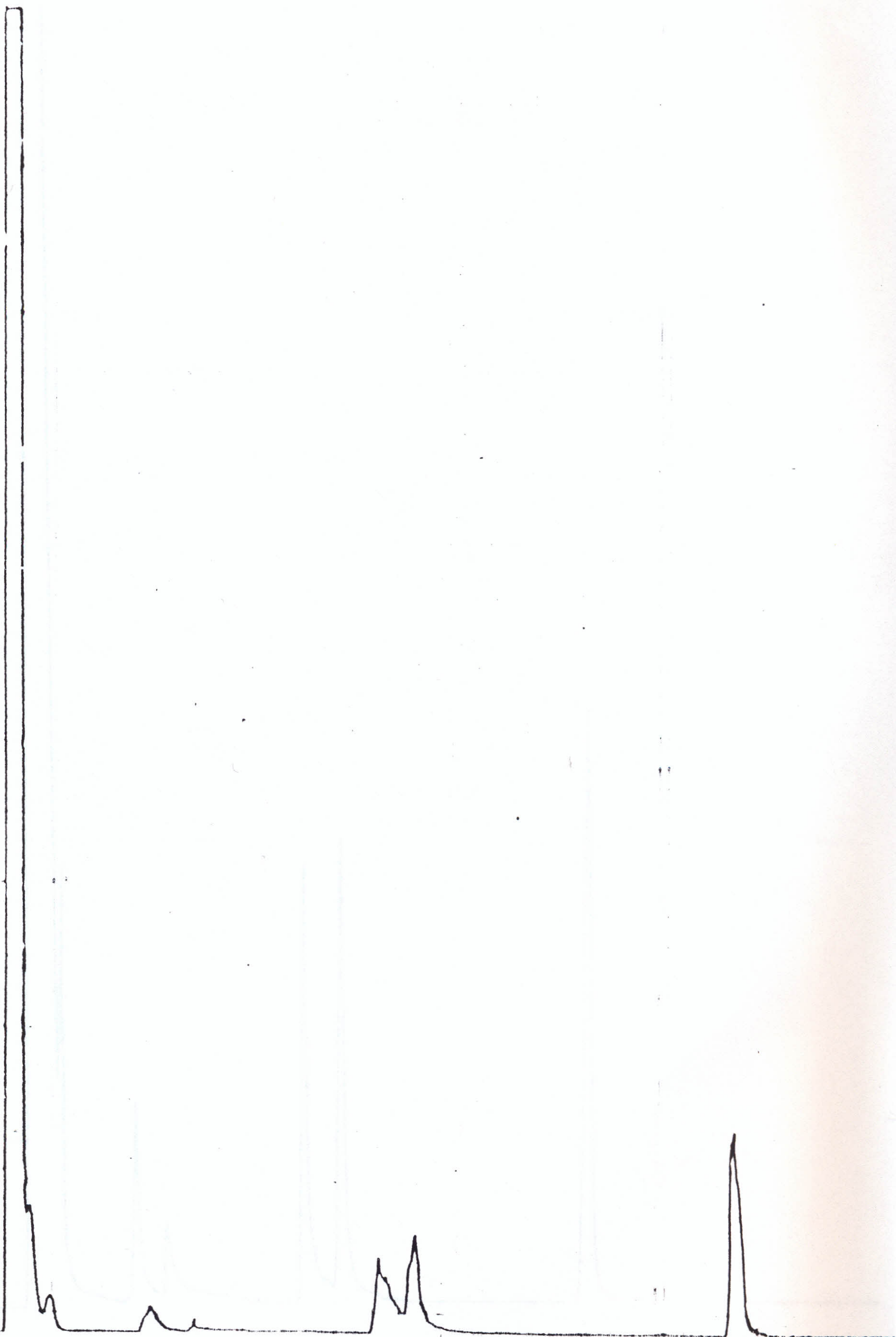


ure 4.5 The manufacturer's capillary column performance with the test sample TRF BP5



91 .

1. SOLVENT 2. DECANE 3. 4-CHLOROPHENOL 4. DECYLAMINE 5. UNDECANOL
6. BIPHENYL 7. PENTADECANE



4.6: : Sample of results obtained when testing column performance using the test sample.

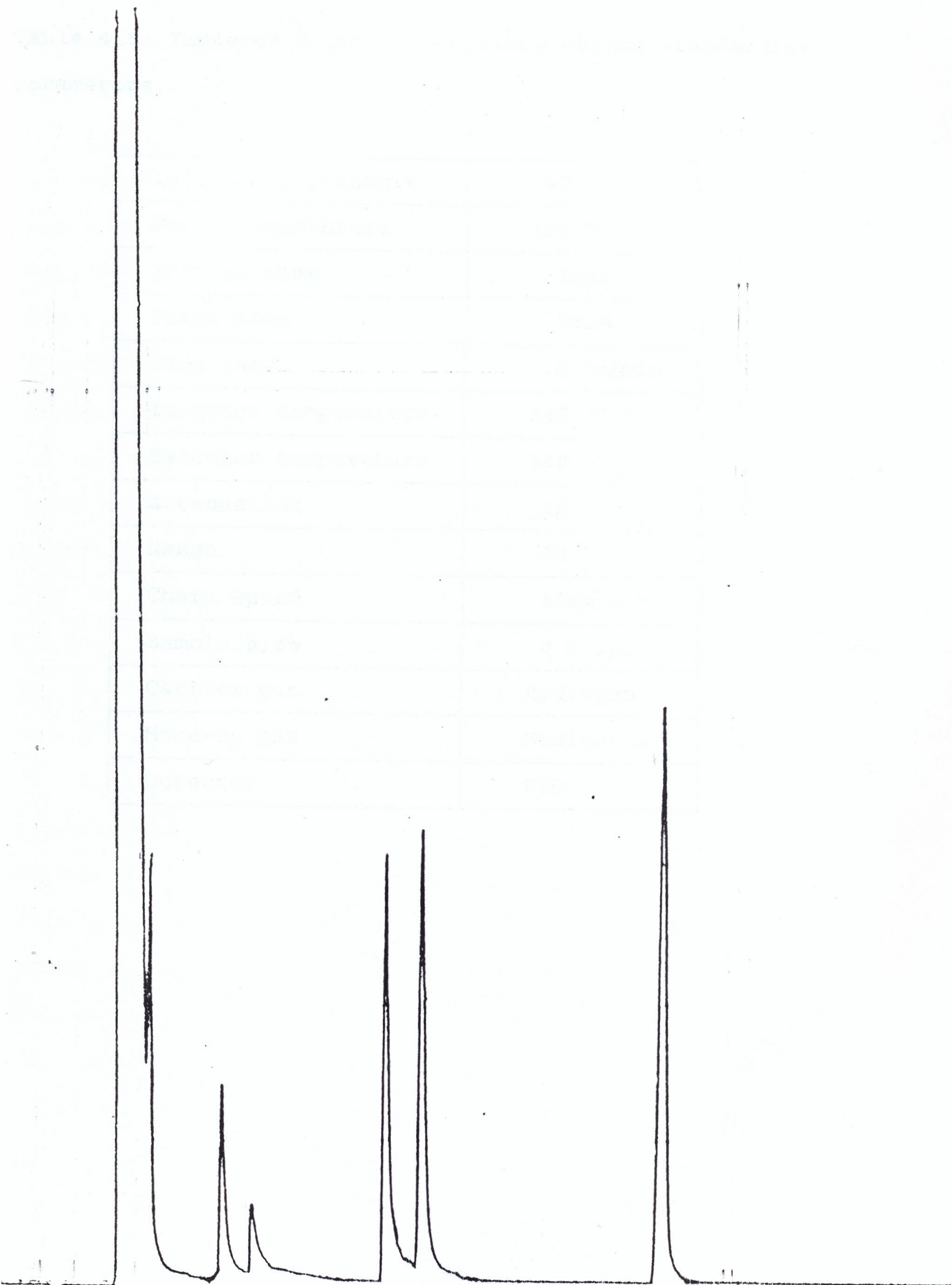


Fig. 4.7 : Sample of peak obtained when using H_2 as carrier gas and N_2 as make up gas.

Table 4.5: Table of Sigma 3B capillary column standardized parameters.

Initial temperature	40 °C
Final temperature	190 °C
Initial time	1min
Final time	5min
Ramp rate	5 °c/min
Injector temperature	240 °C
Detector temperature	380 °C
Attenuation	16
Range	10
Chart speed	10mm/min
Sample size	≤ 0.1μl
Carrier gas	Hydrogen
Make-up gas	Medical air
Detector	FID

4.2.0 Selection of a suitable solvent for use in the extraction of the coffee samples.

The Pure TCA was found to dissolve readily in propan-2-one, hexane and dichloromethane. Any of the three solvents could therefore be used for the extraction of the coffee samples. In order to choose the best extraction solvent, two important factors were investigated. First, the detection limit of TCA in the three solvents was tested using the GLC and TLC. Secondly, the solvent which could recover most TCA from a known spike of an untainted coffee sample. The results of these investigations are discussed in the following section.

4.2.0.1 Detection limits of the TCA in dichloromethane, hexane and propan-2-one using GLC and TLC.

The minimum amount of the 2,4,6-TCA that can be dissolved in dichloromethane and give a reasonable peak (1.2cm high) in the GLC was 1 x 10^{-6} $\mu g/\mu l$. A similar amount in hexane was also detectable using the GLC, though the peak was very small and needed peak enhancement for proper identification. A test of the same amount of the TCA in propan-2-one yielded chromatograms which were not easily interpretable. This was because propan-2-one is a solvent capable of dissolving a wide range of compounds. Therefore, up to this stage it was found that either hexane or dichloromethane could be used in this study, but the

dichloromethane solvent was selected for the extraction of the coffee samples for two reasons: First, it gave a good peak and a clean chromatogram and secondly, it recovered most of the spike from the spiked coffee sample.

The detection limit of TCA using thin layer chromatography was measured in the hope that it could be sufficiently low to be used to confirm the detection by GLC. It was found that the minimum amount of the TCA that could be detected was 1.8μg/μl. This detection limit by comparison, was much higher than that of GLC, such that even the coffee samples which were found to be highest in TCA could not be detected by the TLC.

Spadone[38] *et al.* in their analytical investigation of Rio off-flavour in green coffee, also used dichloromethane as solvent for their extractions and were able to identify TCA in their samples in concentrations ranging from 1-100 ppb. Investigations about the TCA carried out on other foods reported the use of different solvents with yields of different concentrations: wine - Pentane-ethyl acetate(3:1), 20-370 ng/L[30]; cocoa - dichloromethane, 2μg/kg[28] and dried fruit - 10% diethyl ether/pentane, 0.2-12 μg/kg[25]. The next section describes how the identity and the purity of TCA which was successfully prepared in the laboratory was confirmed.

4.2.1 Preparation of TCA.

The commercially available sample of TCA was small. Therefore, it was necessary to prepare an additional sample by methylation of TCP. The yield of the TCA (8.04g) which was prepared was adequate for this study. The melting point of the prepared compound was between 61-64 °C after complete drying, and that of the commercial TCA was 61-62 °C[61]. From these melting point values, it looks like the prepared compound was relatively pure but could contain some impurities responsible for the increased melting point range. Dry equimolar mixtures of the commercial TCA and the prepared TCA melted between 61-62 °C and that of the prepared sample and an impurity (TCP) which was added melted between 45-49.5 °C. The compound prepared was judged pure TCA since the melting point of the two mixtures was not changed and occurred in about the same range. The melting point of the prepared sample was depressed by the impurity (TCP) as was expected.

A solution of the product remained yellow when treated with alcoholic ferric chloride solution while, a TCP solution turned blue. This is a known characteristic of phenols, and indicated that the phenol used in the preparation of the product was fully methylated. Both TLC and GLC results confirmed the prepared compound was TCA. A TLC examination of the commercial and the prepared sample gave similar R_f (0.71) values and examination by GLC gave similar R_t (1.55 min). From the confirmatory tests

carried out, it was concluded that the compound was successfully prepared . The following section 4.2.2 discusses the results obtained when an attempt was made to extract the active ingredient of the fungicide Prochloraz (also known as Octave).

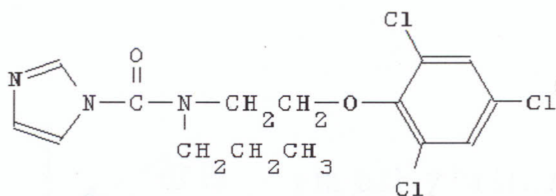
4.2.2 The Prochloraz extract

After extraction with dichloromethane solvent, the Prochloraz extract was cleaned using column chromatography with hexane as a solvent. During the elution process, samples (50ml) were collected and concentrated *in vacuo*. The concentrated eluates were examined by TLC using hexane. However, no material was observed to have been eluted with hexane. The polarity of the column solvent was increased using a mixture of hexane:chloroform (3:1). A band which was observable in the lower part of the column was eluted. After the use of about 50ml of the solvent system, no further change was observed on the column except that the sample appeared to turn dark. The change was thought to be due to a reaction taking place or to some optical phenomenon occurring.

The TLC spots of the Prochloraz fractions when investigated showed only one spot. This indicated that the fraction was pure and could be combined. Evaporation yielded crystals again suggesting that the fraction was pure. The fractions of the eluate from hexane:chloroform (3:1) represented the extractable

component of the commercial Prochloraz powder. Examination of the extract showed that it did not contain any TCA as the GLC chromatograms of the fraction (R_t .38min.) were different from that of the pure TCA (R_t 1.55min.). Examination of the extracted coffee samples for the presence of the Prochloraz did not show any positive results. This observation could possibly suggest that this examination was carried out long after the spraying of the fungicide had been done. Alternatively the fungicide completely gets broken down without significant residues.

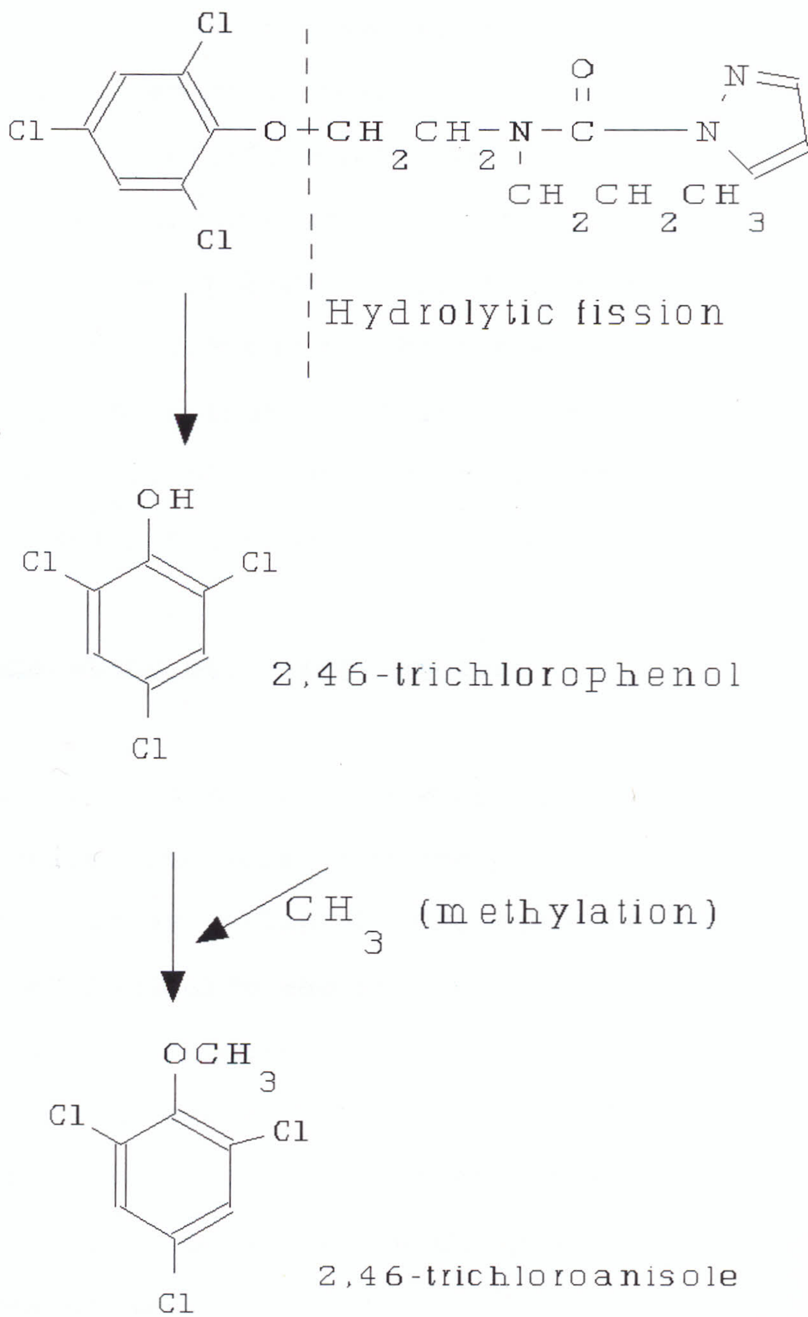
Prochloraz is an organic fungicide of the imidazole class. When combined with manganese dichloride it forms a complex, which is marketed under the name of OCTAVE. A close look at the structure of the fungicide reveals that the Prochloraz has the chemical structure;



The structural formula of Prochloraz

Prochloraz does not possess truly systemic properties, but does show some translaminar mobility. This compound could be a potential taint facilitator since examination of its structure, suggest degradation of the compound stands a chance of yielding a TCP among the potential metabolites. Trichlorophenols are readily transformed to the corresponding trichloroanisole *in vivo* according to the reaction:

Fig 4.3.0 Probable route for Prochloraz degradation



However, it is also worth noting that there are endogenous chlorophenols on the bean surface from which micro-organisms can form TCA as put forward by Amorin[64]. Whether these chlorophenols are found naturally in the bean or are the products of the transformation of phenolic bean substrates is a matter of speculation. But the coffee bean does contain a lot of phenolic substances[64]. This could mean coffee from any part of the world, with or without the application of octave could be capable of exhibiting the off-flavour if these phenolic substances which participate in the formation of the off-flavour are present. So far it is only Rio off-flavour[38] which has been extensively reported as having the problem.

4.3.0 **Preparation of coffee samples.**

Ground coffee samples were stored in a deep freezer to prevent volatile materials from escaping. The untainted coffee sample identified as 'clean' AC0038 was obtained from areas where the Prochloraz fungicide was not in use, and this was the sample which was used in the selection of dichloromethane as the solvent for use in the extraction process. The following section discusses the results obtained after running different standards in the GLC in order to ascertain the position and the R_t of TCA in the chromatogram.

4.3.1 Results of the investigations of the Retention times(R_ts) of the taint TCA

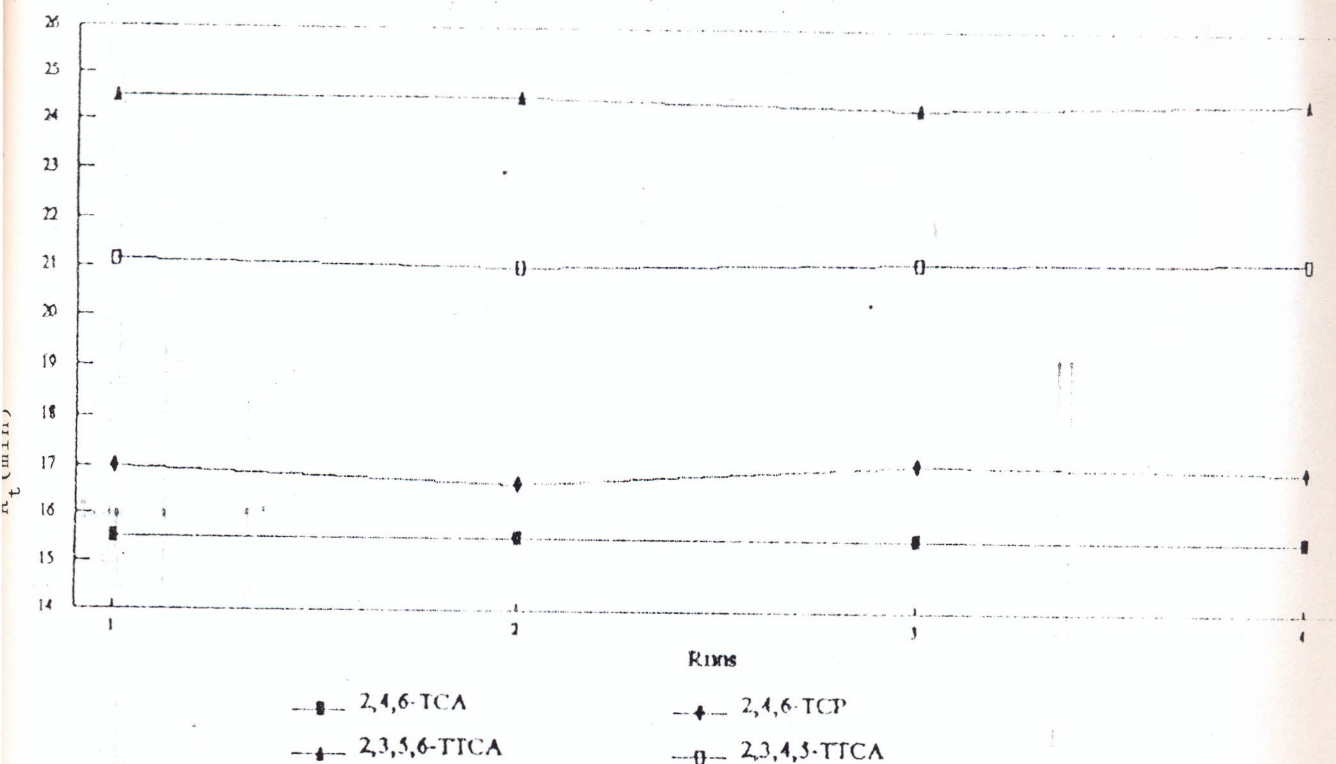
The standards of pure samples of TCA, TCP, 2,3,5,6-TeCA and 2,3,4,5-TeCA were run on the GLC to determine their relative positions and retention times in the chromatogram (Table 4.6). The retention times of TCP, 2,3,5,6-TeCA and 2,3,4,5-TeCA were used to determine the position of the TCA on the chromatogram which could also be reproducible. The retention times were measured from the solvent peak base, since the consistency of injecting the sample in the column and simultaneous pressing of the start button to start the temperature programming could not be harmonised. These retention times were then used to check for the presence of TCA and the other standards in the coffee samples. The results indicate that the mean and the standard deviation of the R_ts of TCA was 15.55$\pm$0.00 min, TCP; 16.94$\pm$0.17 min, 2,3,5,6-TeCA; 24.45$\pm$0.09 min and 2,3,4,5-TeCA; 21.13$\pm$0.07 min. The results had little or no deviations from the mean values. Standard deviation (S.D) is an important statistical measure for the quality of data. The smaller the S.D numerically the more precise the data and presumably the more accurate the data. Therefore, the S.D is a statistical method used for evaluating the reliability of data[72] .

The reliability of the R_ts obtained in Table 4.6 are further confirmed by the graphs in Fig. 4.8 which show that the R_ts are almost constants as they have almost zero gradients.

Table 4.6 Retention times (R_t) of the individual standards:

	Retention time (min)				Mean ± S.D.
Sample	1	2	3	4	
TCA	15.55	15.55	15.55	15.55	15.55±0.00
TCP	17.00	16.65	17.10	17.00	16.94±0.17
2,3,5,6-TeCA	24.50	24.50	24.30	24.50	24.45±0.09
2,3,4,5-TeCA	21.15	21.00	21.15	21.20	21.13±0.07

Fig. 4.8 Graph of the Retention times (R_t) of the individual standards against the runs:



Equimolar samples of the standards containing 5x 10^{-3} $\mu g/\mu l$ each were mixed and their retention times recorded (Table 4.7a). The ratios of these R_ts were calculated and used for quick interpretation of the results. The ratio of TCA in relation to other standards was obtained by taking the retention times of TCA and dividing by that of other standards (Table 4.7b). This was done as the pure standards when mixed together would interfere with each other due to the possible intermolecular interactions. The reproducibility of the results was also checked by repeating the experiment after 24 hours (Table 4.8) and 4 days (Table 4.9) from the first run. Graphs of the R_ts against the runs were plotted and the change in the gradients of the line graphs interpreted.

Table 4.7 a : R_ts of the chromatogram of the mixed standards.

Retention time (min)					Mean ± S.D.
Sample	(First run)				
	1	2	3	4 *	
TCA	15.50	15.30	15.70	12.25	15.50±0.16
TCP	16.50	16.35	16.80	13.20	16.55±0.19
2,3,4,5-TeCA	20.70	20.55	21.00	17.30	20.75±0.19
2,3,5,6-TeCA	24.20	24.10	24.45	20.75	24.25±0.15

*** Rejected as an outlier**

Table 4.7 b: Ratios of the R_ts from Table 4.7 a.

Retention time (min)				Mean±S.D
Sample	(First run)			
	1	2	3	
TCA	1.00	1.00	1.00	
TCP	0.94	0.94	0.93	0.94±0.01
2,3,4,5-TeCA	0.64	0.63	0.64	0.63±0.02
2,3,5,6-TeCA	0.75	0.74	0.75	0.74±0.02

Fig. 4.9 Graph of Retention times of the individual standards against runs.

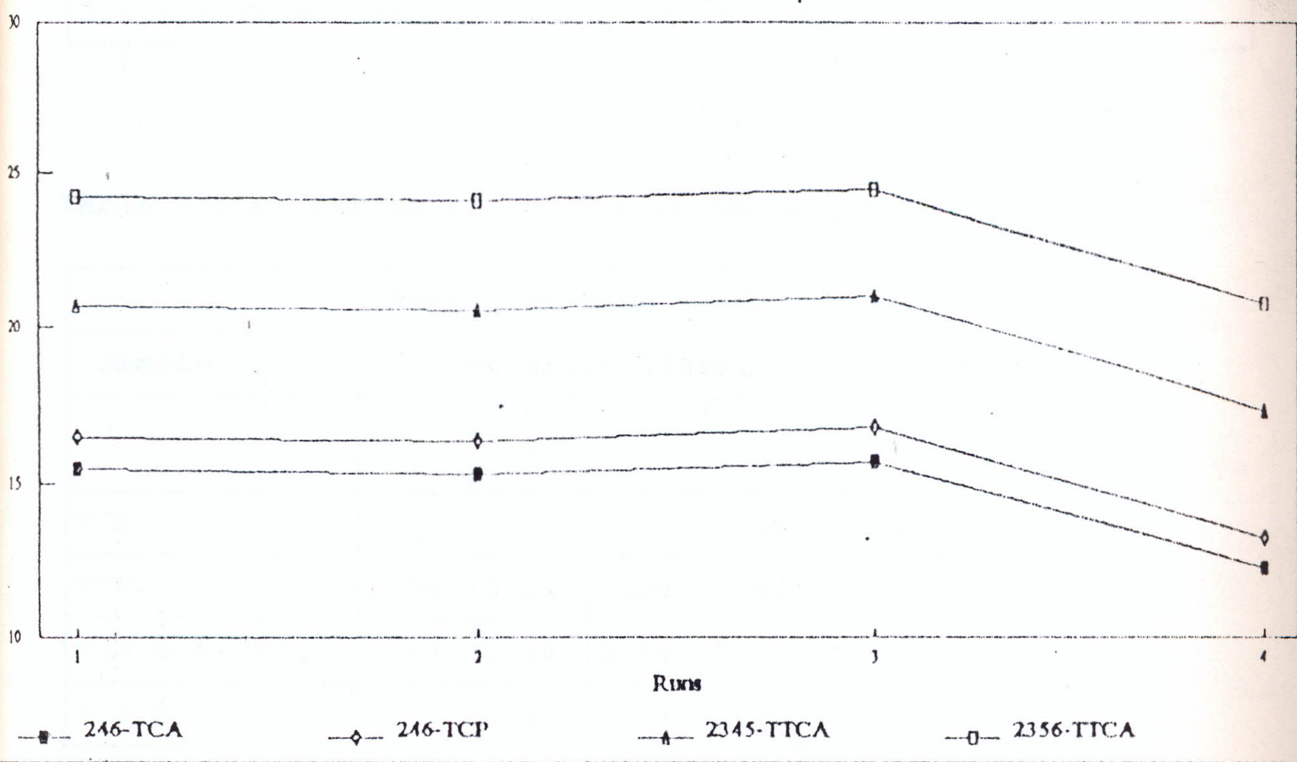


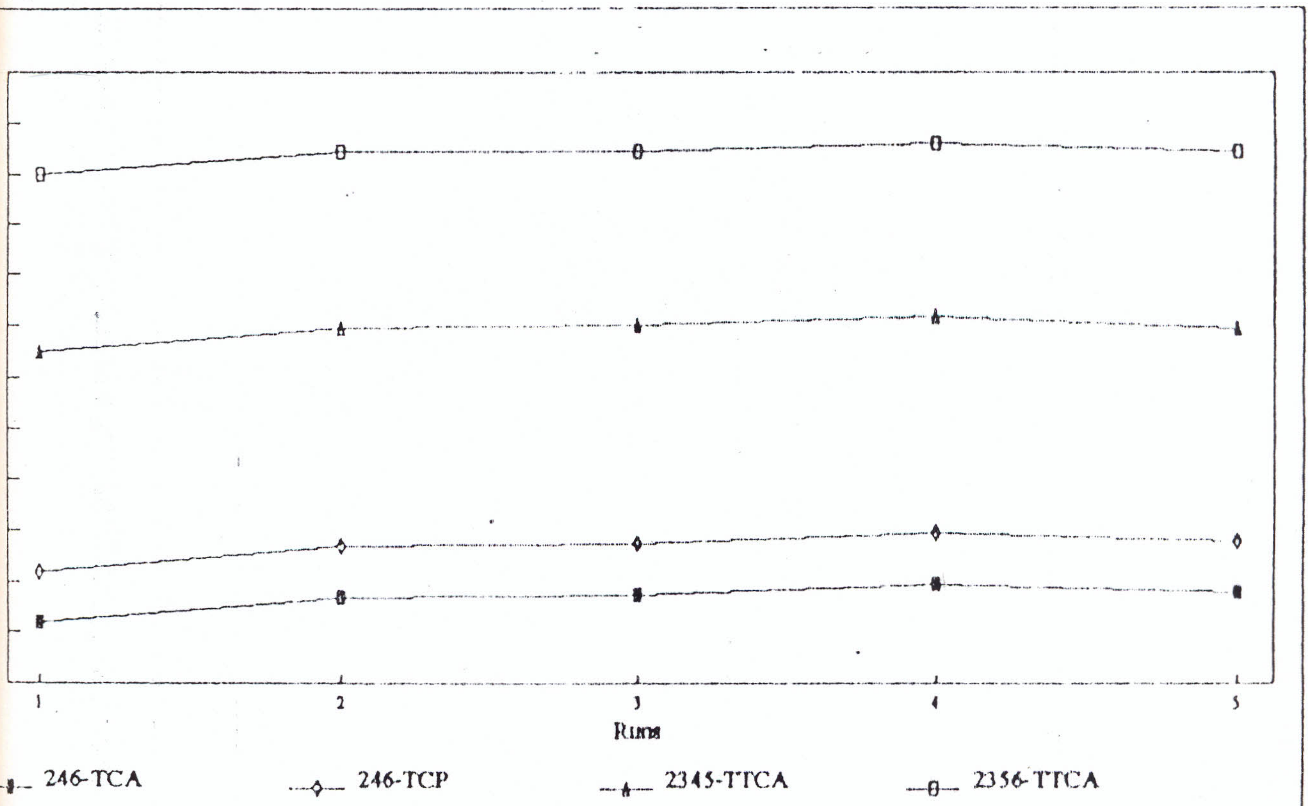
Table 4.8 a : R_ts of the chromatogram of the mixed standards run after 24 hours.

Sample	Retention time (min) (Run after 24Hrs.)					Mean±S.D
	1	2	3	4	5	
TCA	15.20	15.70	15.75	15.95	15.80	15.68±0.25
TCP	16.20	16.70	16.75	16.95	16.80	16.68±0.25
2,3,4,5-TeCA	20.50	20.95	21.00	21.15	20.95	20.91±0.22
2,3,5,6-TeCA	24.00	24.45	24.45	24.60	24.45	24.39±0.20

Table 4.8 b: Ratios of the R_ts in Table 4.8 a.

Sample	Retention time (min) (Run after 24Hrs.)					Mean±S.D
	1	2	3	4	5	
TCA	1.00	1.00	1.00	1.00	1.00	
TCP	0.94	0.94	0.94	0.94	0.94	0.94±0.00
2,3,4,5-TeCA	0.63	0.64	0.64	0.64	0.65	0.64±0.01
2,3,5,6-TeCA	0.74	0.75	0.75	0.75	0.75	0.75±0.00

Fig. 4.10 Graph of Retention times of the individual standards against runs.



The results show that the position of TCA relative to the other pure standards was reproducible. The mean of the R_t obtained was averagely 15.55 min with the standard deviation of ±0.16. The graph obtained (Fig. 4.10) was almost straight line graphs. It was deduced that the results obtained were valid, so the retention times of the chemical standards obtained could be relied upon and used for reference as standards.

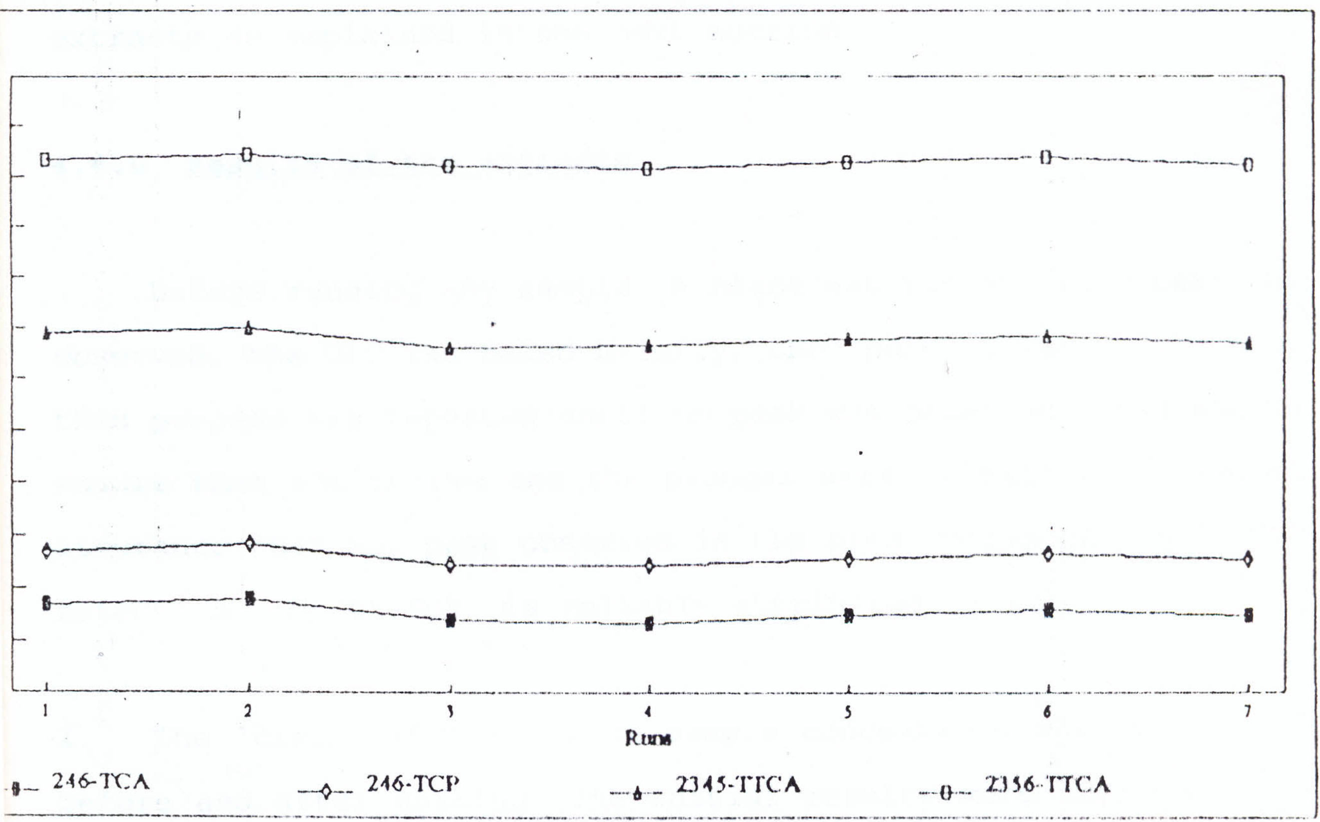
Table 4.9 a: R_ts of the mixed standards (Run after 4 days).

Sample	Retention time (min)							Mean±SD
	1	2	3	4	5	6	7	
TCA	15.70	15.80	15.40	15.35	15.50	15.60	15.50	15.55
TCP	16.70	16.85	16.45	16.45	16.60	16.70	16.60	16.62
2,3,4,5-TeCA	20.90	21.00	20.60	20.65	20.80	20.85	20.70	20.79
2,3,5,6-TeCA	24.35	24.45	24.20	24.15	24.30	24.40	24.25	24.30

Table 4.9 b: Ratios of the R_ts of the mixed standards (Run after 4 days).

	Retention time (min)							
Sample	1	2	3	4	5	6	7	Mean±SD
TCA	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1
TCP	0.94	0.94	0.94	0.93	0.93	0.93	0.93	.93±.005
2,3,4,5-TeCA	0.75	0.75	0.75	0.74	0.75	0.75	0.75	.75±.003
2,3,5,6-TeCA	0.64	0.65	0.64	0.64	0.64	0.64	0.63	.64±.005

Fig. 4.11 Retention times of the individual standards against Runs.



The runs of the chemical standards after 4 days was done repeatedly and the R_ts obtained were found to fall within the ranges of the first two runs performed earlier.

The results of the retention times show that the position of TCA in relation to the other standards was always similar, though the actual position of appearance depends on the solvent base which was the reference of the measurements. The results also show that the retention times of the mixed standards are reproducible and could be used reliably. These retention times were used as reference retention times during the run of the coffee samples extracts as explained in the next section.

4.4.0 Results of the extracts

Before running any sample, a blank was run and if a peak was observed, the GLC was baked briefly, then pure solvent run again, this process was repeated until no peak was observed. This was to ensure that the column and the plunger were clean. This gave an assurance that any peak observed in the area corresponding to the retention time of TCA is reliably attributed to TCA.

I. The 'clean' AC0038 coffee sample concentrate was run on GLC before and after spiking. The initial results were obtained from the run of the non-spiked sample. Four different runs were performed yielding four sets of results (Table 4.10). The results had two distinct peaks of similar R_t to TCA. The retention times

of the peaks were measured from the base of the solvent peak, such that the immediate peak after the solvent peak was peak 1 and the second, peak 2 consecutively (Table 4.10 Result I). The possible presence of TCA was tested in the 'clean' sample. The sample was injected with 0.025μl (100ppm) of TCA and no peak enhancement was observed indicating the absence of the taint. (Table 4.10 Result II). Instead a third peak, that of the TCA appeared.

Table 4.10: The GLC profile of 'clean' AC0038 concentrate

Retention times (Min)					
Peak	**(Result I)**				**Mean±SD**
	1	2	3	4	
1	6.40	6.40	6.60	6.70	6.53±0.13
2	10.35	10.30	10.65	10.70	10.50±.18
Ratio(1/2) 1	1.00	1.00	1.00	1.00	
2	0.62	0.62	0.62	0.63	0.62±0.0

When the results of the GLC profiles of the coffee extracts (in appendix III) were compared to the results obtained by Spadone[38] *et al.*, few peaks were observed. This could be due to the experimental conditions used which were different. For example the GLC sensitivity used was low compared to that used by Spadone[38] *et al.*. An attempt to increase the sensitivity of the Sigma 3B GLC

yielded a lot of peaks with excess background noise. Again this just meant that the established standardized parameters from Table 4.10 were the best for use in this study.

II. A concentrate of 'clean' AC0038 was spiked with TCA and run. Three sets of results from the three different runs showed three distinct peaks (Table 4.11 result I). When a 100ppm of TCA was added to the sample and GLC run, peak 3 was strongly enhanced indicating the presence of TCA (Table 4.11 Result II). The peak enhancements were deduced by calaculating the ratios of the peak heights. Thus the results indicate that the 'clean' coffee sample was successfully spiked, and the standard used as a spike was extracted with the solvent used for extraction. The presence of TCA found only after a spiking had been done served as a strong base to use for the search of the taint in the suspected coffee samples.

Table 4.11: The GLC profile TCA spiked 'clean' AC0038 concentrate.

Retention times Min)					
Peak	**(Result I)**			**Mean±S.D**	**(Result II**
1	0.50	0.50	0.50	0.50±0.00	-
2	6.20	6.50	6.65	6.45±0.19	6.75
3	15.10	15.50	15.65	15.42±0.23	15.80*
Ratios (2/3) 2	1.00	1.00	1.00		1.00
3	0.41	0.42	0.42	0.42±0.00	0.43

*** Enhanced peak**

III. A concentrate of 'clean' AC0038 spiked with three standards namely, TCA, 2,3,4,5-TeCA and 2,3,5,6-TeCA and run in duplicate on GLC gave the results shown in Table 4.12.

Table 4.12 a: GLC profile of the spiked 'clean' AC0038 concentrate.

	Retention times (Min)		Mean±S.D
Peak	1	2	
1	6.30	6.60	6.45±0.15
2	10.50	10.60	10.55±.05
3	15.50	15.70	15.60±.10
4	20.70	20.90	20.80±.10
5	24.20	24.40	24.30±.10

When the results obtained above were compared to the established retention times of the chemical standards (Tables 4.6 - 4.9), all the three standards used in the spiking process were deduced to be present. Again there was an indication here that the clean coffee sample was successfully spiked and the extraction solvent was able to recover the spikes. This was a further hope strengthening the basis to be used for the taint analysis in the coffee samples. The result was indeed encouraging for the study as the spiking solutions used were containing 5 x 10^{-3} μg/l, a reasonably low quantity of the taint for the spike. Section 4.4.1

reports the analysis of the results obtained when the peaks obtained were investigated to find if they corresponded with any of the standards. The results were positive as confirmed by the peak enhancements observed in results i,ii,iii and iv of this section. An eventual investigation of all the four standards gave similar results.

Table 4.12 b: Ratios of the R_ts of the GLC profile of the spiked 'clean' AC0038 concentrate.

Ratios of peak 3 divided by the rest			
Peak	I	II	**Mean±S.D**
3	1.00	1.00	
4	0.75	0.75	0.75
5	0.64	0.64	0.64

4.4.1 Confirmation of the peaks by way of peak enhancement

(**i**) When 100ppm of TCA was added, a peak with retention time 15.75 min was enhanced as shown in Table 4.13a.

Table 4.13 a: The enhanced GLC profile of the spiked 'clean' AC0038 concentrate when 100ppm of TCA was added.

Peak	Retention times (Min)	Ratios
3	15.75 (enhanced)	1.00
4	21.00	0.75
5	24.75	0.64

(ii) 100ppm of 2,3,5,6-TeCA was also added. A second peak was observed to have been enhanced (Table 4.13b).

Table 4.13 b: The 2,3,5,6-TeCA enhanced GLC profile of the spiked 'clean' AC0038 concentrate when 100ppm of 2,3,5,6-TeCA was added.

Peak	Retention times (Min)	Ratios
3	16.00 (enhanced)	1.00
4	21.20	0.75
5	24.15 (enhanced)	0.66

(iii) 2,3,4,5-TeCA was finally added. A third peak was enhanced (Table 4.13c).

Table 4.13 c: The 2,3,4,5-TeCA enhanced GLC profile of the spiked 'clean' AC0038 concentrate when 100ppm of 2,3,4,5-TeCA was added.

Peak	Retention times (Min.)	Ratios
3	16.00	1.00
4	21.20	0.75
5	24.70	0.65

The results strongly indicate that peak 3 was probably due to TCA; peak 4, 2,3,5,6-TeCA and peak 5, 2,3,4,5-TeCA.

(**iv**) Concentrate of clean AC0038 spiked with TCA, TCP, 2,3,4,5-TeCA and 2,3,5,6-TeCA was run on the GLC in duplicate. Five distinct peaks were obtained (Table 4.14).

Table 4.14 a: GLC profile of the 'clean' AC0038 concentrate spiked with 100ppm of TCA, TCP, 2,3,4,5-TeCA and 2,3,5,6-TeCA after extraction.

Retention times (Min)			Mean±S.D	Ratios		Mean±S.D
Peak	1	2		1	2	
1	6.00	6.70	6.35±0.35	-	-	
2	9.50	10.60	10.05±0.55	-	-	
3	14.65	15.60	15.13±0.48	1.00	1.00	
4	15.70	16.65	16.18±0.48	0.93	0.94	0.94±0.01
5	19.90	20.80	20.35±0.45	0.74	0.75	0.75±0.01
6	23.40	24.30	23.85±0.45	0.63	0.64	0.64±0.01

(i) When 100ppm of TCA was added, the peak 2 was enhanced. indicating that this peak is due to the recovered spike of TCA (Table 4.14b).

Table 4.14 b: The TCA enhanced GLC profile of the spiked 'clean' AC0038 concentrate when 100ppm of TCA was added to check for an enhancement.

Peak	Retention times (Min) (x)	Ratios (3/x)
1	6.75	
2	10.80 (enhanced)	
3	15.90	1.00
4	16.90	0.94
5	21.15	0.75
6	24.60	0.65

(ii) When 100ppm of TCP was added, peak three also got enhanced.

Table 4.14 c: The TCP enhanced GLC profile of the spiked 'clean' AC0038 concentrate when 100ppm of TCP was added to check for anenhancement.

Peak	Retention times (Min) (x)	Ratios (3/x)
1	6.85	-
2	10.75	
3	15.85	1.00
4	16.90 (Enhanced)	0.94
5	21.10	0.75
6	24.60	0.64

The above preliminary studies led to the conclusion that the retention time of TCA on the transformed GLC when the standardized conditions are used was 16±0.5 min., 2,46-TCP, 17±0.5 min, 2,35,6-TeCA, 22±0.5min and 2,3,4,5-TeCA, 24±0.5 min. All these results put together lay a reasonably strong foundation for the analysis of the taint, TCA in the coffee samples. The retention times from the chromatograms obtained after running the purified extracts of the coffee samples were obtained. These retention times were carefully interpreted against those of the pure standards obtained from the preliminary tests done. The retention times which differed by ±0.5min were taken to be within the limit of the positive results, a factor which was determined during the preliminary investigations. The established results were tabulated as below (Table 4.15):

Table 4.15: Results of the coffee samples checked for the presence of TCA.

SAMPLE DESCRIPTION	PRESENT (YES) ABSENT (-)	OCTAVE USED
Firsts cos3 6.10.93	-	
Lights cos15	-	
Firsts octave cos13	YES	YES
Firsts Good 10.2.94	-	
Seconds Good cos4	-	
Firsts octave cos6 8.12.93	YES	YES
93	-	
Octave + Cu 15.5 kg/ha	YES	YES
Firsts Ruiru 11 cos202 6.10.93	YES	NO
Firsts Good cos4 15.10.93	-	
Firsts Good cos10 26.	-	
Seconds cos2 29.9.93	-	
Firsts octave cos9 19.1.94	YES	YES
Firsts Good cos5 22.11.93	-	
CBK coffee dispatched Aug 92-Jan 93	-	
Firsts Good cos7 22.12.93	-	
Ruiru 11 cos16 3.94	-	
Firsts Good cos11 2.2.94	-	
Lights cos8	-	
Shirlan 0.75L + Cu 5.5 kg/L	-	
Firsts Good cos1 1993	YES	NO
Lights cos2 29.9.93	-	
Daconil 2.2 kg + Cu 5.5 kg/ha	-	
Seconds cos14 23.2.94	-	
Clean AC0038	-	
AC0038 parchment	YES	YES
AC0038	YES	YES

CHAPTER 5

5.0 Comments and conclusions

The main purpose of this study was to investigate the relationship between the Prochloraz fungicide and the reported taint characteristic observed in some coffee batches. Circumstantial evidence tended to implicate Prochloraz as the fungicide responsible for the taint. This study was provoked by the work of Ojijo N.K.O. *et al.* who did an exploratory study of the occurrences of off-flavour in some of the Kenyan coffee. The study showed that the TCA which is associated with the taint was present in all the coffee samples from the areas where the octave was used (Table 4.15). All the six areas where the fungicide was used showed presence of the taint. In addition to these, the other coffee samples namely, Firsts Ruiru 11 Cos 202 6.10.93., and two of the three types of the AC0038 coffee samples showed the presence of the taint. The possible reason to this could be some of the chemicals used during field maintenance which are chlorinated. The off-flavour occurrences is not a new problem as it had already been reported to occur in a number of different foods[21,22,23,24,25], and in Rio coffee from Brazil[38]. An extensive review of TCA occurrence and formation pathways was presented by Maarse *et al.*[27]. In most of the cases reported the occurrences of the taint appeared to be related to some form of industrial contamination, for example, water chlorination and use of chlorinated pesticides or fungicides. These primary contaminants would undergo various chemical or microbial

degradation lead to produce amongst others, TCP[27,65] which would then be converted into the corresponding TCA[23,45]. It is unlikely that the Rio taint observed in the Brazilian Rio coffee was a result of TCA produced by industrial contamination. This is because the taint was so widespread amongst Brazilian coffee (some 20% of the coffee produced is reputed to carry the taint). It was been suggested that TCP is produced by fungi growing on the Brazilian coffee and that this is converted to TCA by the same or other organism[70,71].

Therefore, the coffee samples found tainted from Table 4.15 show high degree of relationship with the octave since all the coffee treated with the fungicide gave positive result for the presence of the taint. A discussion of the possible degradation of the Prochloraz is presented in section 4.2.2. It was seen that a potential taint producer of the fungicide could be due to the metabolite TCP. This metabolite could be the direct precursor of the TCA found in the tainted coffee samples, otherwise the origin of TCP is not easily speculated. Therefore, TCA and TCP being known metabolites of the semi-systemic Prochloraz, it is not unexpected to find these metabolites in the coffee which was exposed to the octave. Although it is possible that TCA and TCP may be present in coffee as a result of fungal activity, this has not been confirmed. The purpose of using fungicides is to control fungal growth. It would, therefore seem unlikely that TCA and TCP would be produced as a result of fungal metabolism on coffee that has been subjected to a spray regime that is designed to prevent fungal growth. A number of mould strains have been known to be capable of producing

chlorinated metabolites[66,67] and some of these metabolites have been reported as structurally close to the various chlorinated compounds used against pests and diseases[68,69]. TCP might be a natural metabolite of the moulds infesting the coffee beans and the presence of the fungicide Prochloraz provides favourable conditions for the growth of the moulds which uses the TCP in its detoxification process. The moulds then produces the TCA during this process of metabolites conversions which then percolates inside the coffee berries producing the taint.

From this study it can be concluded that the fungicide Prochloraz is associated with the taint in the areas where they were observed in the coffee samples and its use should be discontinued. It can be strongly suggested that an alternative to the fungicide Prochloraz should be used and further research carried out to evaluate further existence of the taint so as to confirm the role of the fungicide prochloraz on the taint production.

Recommendations for further study:

1. The frequent occurrence of a taint similar to the Rio taint in Kenya coffee can damage its reputation as a high quality coffee. Since it is possible that a fungi which contain chloroperoxidase may produce TCA, it may be worthwhile screening all the fungi known to grow on Kenya coffee for

chloroperoxidase activity[67]. There is a report that the causitative agent for warty disease, *Botrytis cinerea*, contains a chloroperoxidase enzyme. It would be sensible, therefore, that when *Botrytis* is observed, non chloride containing fungicides such as copper oxide be used to reduce the possibility of TCA formation.

2. It is also worth investigating the actual presence of TCP on the coffee berries in areas where tainted coffees were obtained, the method for the work can be traced as such an investigation was reported to be underway by Spadone *et al.* [38]. This when related to the micro-organisms which might be found present may shed more light on the origins of the observed coffee taint.
3. With the emergence of capillary columns and increase in resolutions in GLC, there is a strong need to develop a Chemical method for assessing the coffee quality using this modern technique which will be definitely objective and reliable as it can be reproducible. A further investigation for the presence of TCA in the other crops where Prochloraz is used is also recommended.

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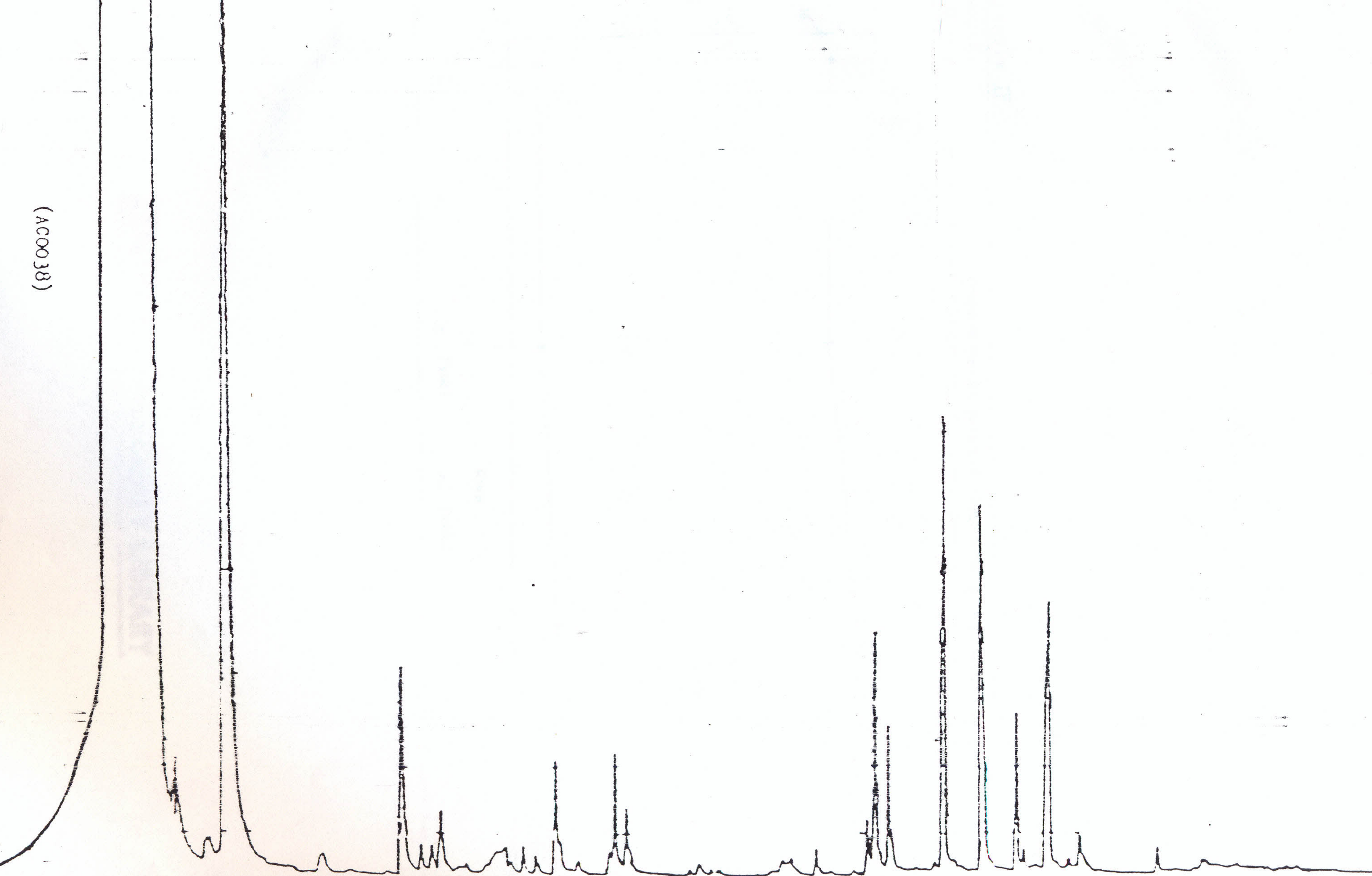
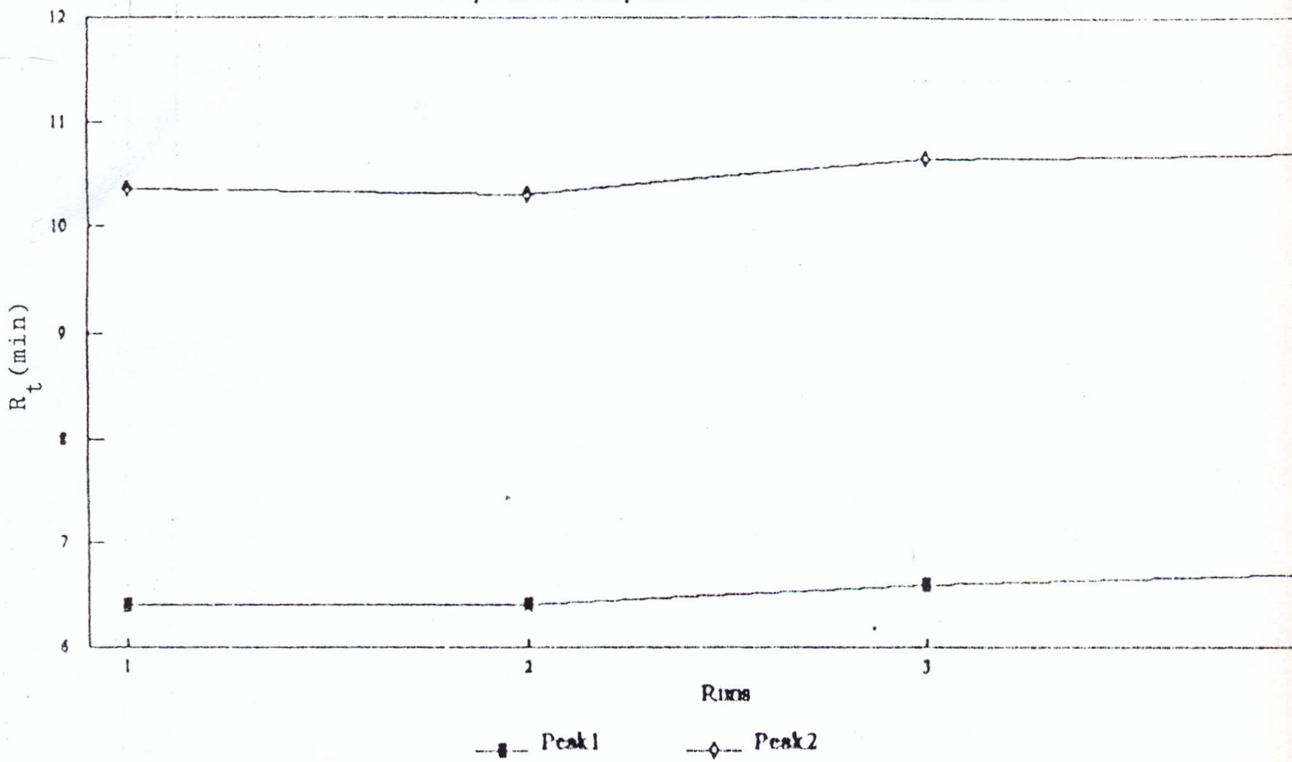
APPENDICES



Appendix I : Sample of chromatograms obtained from coffee samples run on GLC.

Appendix II



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